THE REFRACTORY PERIOD OF THE NORMALLY-BEATING DOG'S AURICLE; WITH A NOTE ON THE OCCURRENCE OF AURICULAR FIBRILLATION FOLLOWING A SINGLE STIMULUS

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Plate 8

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In all previous determinations of the refractory period of the mammalian auricle two factors influencing the accuracy of the measurements obtained have been necessarily present as a part of the technique of the experimental procedure. In order to control the exact time in the cardiac cycle at which the interrupting stimulus is introduced it has hitherto been necessary to drive the heart by a series of break induction shocks at a rate more rapid than that of the spontaneous rhythm. The local stimulating effect of these repeated shocks upon the vagus endings of the myocardium has made accurate measurements possible only upon the atropinized heart, and in many instances has also led to an irregular failure of response at a level above the true refractory period. The occasional interruption of the rhythm by false responses, upsetting the essential order of at least several sequential cycles preceding that one during which the refractory period is determined, has also been unavoidable.

A second cause influencing the accuracy of the results previously obtained has arisen from the fact that it has been impossible to record the refractory period from the exact point of stimulation. In all such experimental observations there has necessarily been a small gap between the stimulating and the recording electrodes. It is a reasonable assumption that changes in this small area of musculature, as a result of the repeated induction shocks, may modify the measurement considered as the true refractory period of the auricular muscle. Even

when using the method first described by Lewis, Drury and Bulger (1) in which the shocks, which control the rhythmic beats of the heart, and those which test the refractory period, enter the muscle at precisely the same place, there is a gap of about 15 millimeters between the stimulating and the recording electrodes. This procedure has been necessary owing to the danger to the galvanometer string when the stimulating electrodes are placed too close to the leading-off point. As a result what has actually been measured has been the refractory period at the point of stimulation plus the interval required for the excitatory process to spread from this point to the recording electrodes. In the absence of any added experimental procedure, and under conditions in which both the refractory period and conductivity may be altered in the same sense, such results may be at least proportional to the true refractory period. The instant, however, that one attempts observations under the influence of pressure, cold, certain drugs or changes in the hydrogen-ion concentration of a perfusate, the possible effect of conduction with a decrement throughout this gap, can not be avoided (2). Under these circumstances, in some of which the refractory period may be shortened and conductivity remain unchanged or depressed, the error may be considerable.

By means of a balanced circuit with a double induction coil as the source of stimulus (as suggested by Bishop (3) and applied by Gilson (4)) it has been possible, in these experiments, to place the stimulating electrodes actually astride the proximal lead, thus avoiding any gap, and with no more disturbance of the galvanometric record than is shown in the accompanying curves (Fig. 1). Using a specially designed apparatus (See Appendix) the refractory period of the normally beating dog's auricle has been determined in the following manner.

The animals are anaesthetized with morphia (16 mg.) and urethane (1.0 gm. per kilo). The heart is exposed in situ, the pericardium opened and sewn back against the retracted ribs and a ligature is fastened to the tip of the right auricle drawing it toward the left chest wall. The action-current is led off from the auricular appendix by means of kaolin-paste copper-sulphate electrodes, amplified to 12 to 20 volts and applied to a light relay. Activation of this relay releases a pendulum, which, in turn, throws in a single break induction shock at a time interval determined by the position of a trip-switch along its arc, the normal excitatory process alone controlling the timing of the interrupting shock. The arm

bearing the trip-switch carries an accurately calibrated protractor scale etched upon its face. The local effect of a bombardment with induction shocks is thus avoided and there is the added advantage that the R-S interval may be accurately altered or exactly reproduced.

The threshold of current strength was first determined roughly by watching the heart and the galvanometer string, the point at which a response occurred being noted. The current strength was then doubled and a duplicate series of observations recorded from a time interval well above the observed refractory period to one well below. The refractory period was then always checked in the opposite direction; i.e. if the first determination was made with decreasing intervals, a second series was carried out with increasing intervals. It is of interest that the refractory periods so determined checked within a few thousandths of a second.

TABLE I

Refractory Period of the Dog's Auricle. Spontaneous Rhythm. (R-S Intervals in Seconds)

Dog No.	15		22		17	
Auricular rate	170		150		185	
Refractory period	0.1408					
	0.1305	0.1385	0.1245		0.0978	0.1078
	0.1265↓	0.1316↑	0.1190↓	0.1153↑	0.0940↓	0.1008↑
	0.1250	0.1299	0.1159	0.1123	0.0895	0.0924
	0.1245	0.1275	0.1107	0.1100	0.0858✓	0.0878
	0.1210	**0.1207**	**0.1095**	**0.1064**	**0.0781✓**	**0.0790**
	0.1195↓	**0.1144↑**	**0.1067↓**	**0.1037↑**	**0.0740↓**	**0.0774↑**
	0.1160	**0.1097**	**0.0978**	**0.1015**	**0.0685**	**0.0716**
	0.1140	**0.1021**				**0.0652**

↓ with decreasing, and ↑ with increasing, intervals.

In a series of twenty experiments in which the refractory period was measured before stimulation of the vagus, the administration of atropine or adrenalin, values were obtained varying from between 0.0781 and 0.0858 to between 0.1392 and 0.1410. All measurements were made from the appearance of the excitatory process (R), to the onset of the interrupting stimulus (S)—the R-S interval.* The close agreement of the two values obtained by the method outlined is well shown in Table I.

* All measurements were made with a Lucas Comparator.

In ten animals the right vagus was then exposed in the neck and stimulated with a faradic current of sufficient strength to slow, but not to inhibit completely, the spontaneous rhythm of the auricle. Stimulation of the nerve was commenced a few cycles before the introduction of the induction shock and was continued for less than two seconds unless an abnormal rhythm developed. Typical observations are shown in Table II. Stimulation of the vagus brought about a conspicuous shortening of the refractory period. In no

TABLE II

Refractory Period of the Dog's Auricle. Spontaneous Rhythm. Influence of Vagus and Atropine. (R-S Intervals in Seconds)

Dog No.....	19		16		32		29	
Auricular rate.....	156	70*	167	48*	140	168	187	169
	Control	During vagal stimulation	Control	During vagal stimulation	Control	After atropine	Control	After atropine
Refractory period				0.0848				
			0.1418	0.0771		0.1400	0.1065	
	0.1306	0.0806	0.1312	0.0685	0.1044	0.1333	0.1048	0.1210
	0.1270	0.0737	0.1175	0.0608	0.1013	0.1298	0.0985	0.1150
	0.1245	0.0655	0.1063	0.0559	0.0964	0.1280	0.0918	0.1108
	0.1195	0.0595	0.0992	0.0530	0.0950	0.1257	0.0874	0.1067
	0.1182	**0.0486**	**0.0966**		**0.0938**	**0.1225**	**0.0863**	**0.1041**
	0.1170	**0.0437**	**0.0946**		**0.0880**	**0.1214**	**0.0835**	**0.1012**
	0.1124		**0.0890**		**0.0820**	**0.1155**	**0.0790**	**0.0955**
			0.0816				**0.0760**	

* Average rate.

instance did the refractory period under vagus stimulation exceed in length 0.0629 sec. Indeed in six experiments it lay below the minimum range of the stimulating pendulum (0.0475 sec.). These results agree with those of Lewis, Drury and Bulger (1).

The refractory period was measured in seven hearts after the intravenous injection of atropine (0.001 gm. per kg. body weight). Under the action of this drug it was prolonged well beyond the value obtained in the control observations. The average increase in length amounted to about 20 per cent (See Table II).

TABLE III

Refractory Period of the Dog's Auricle. Spontaneous Rhythm. Influence of Adrenalin after Atropine. (R-S Intervals in Seconds)

Dog No.....	21			27		
Auricular rate.....	82*	167	215	135	145	190
	During vagal stimulation	Control	After atropine followed by adrenalin	After atropine	Control	After atropine followed by adrenalin
Refractory period	0.0765		0.0916		0.1312	
	0.0714		0.0887	0.1604	0.1278	0.0903
	0.0670	0.1165	0.0845	0.1587	0.1248	0.0854
	0.0609	0.1142	0.0806	0.1567	0.1215	0.0787
	0.0545	0.1095	0.0752	0.1503	0.1206	0.0739
		0.1063	**0.0710**	**0.1454**	**0.1155**	**0.0701**
		0.1030	**0.0655**	**0.1425**	**0.1105**	**0.0656**
		0.0984	**0.0619**	**0.1412**	**0.1078**	**0.0624**
				0.1365		

* Average rate.

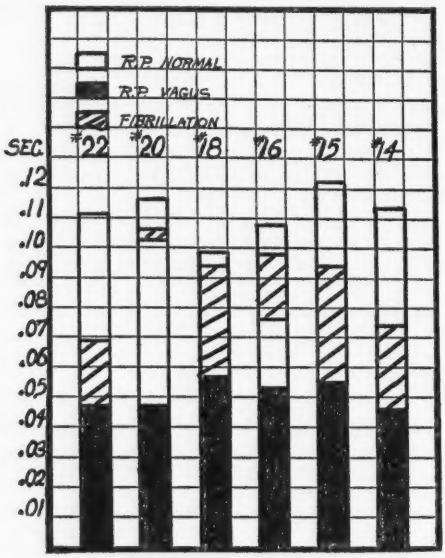
TABLE IV

Refractory Period of the Dog's Auricle. Spontaneous Rhythm. Influence of Vagus, Atropine and Adrenalin. (R-S Intervals in Seconds)

Dog No	20			
Auricular rate.....	120	54*	118	176
	Control	During vagal stimulation	After atropine	After adrenalin
Refractory period	0.1314			
	0.1276	0.0870		0.0906
	0.1238	0.0800		0.0887
	0.1222	0.0670	0.1482	0.0828
	0.1190	0.0540	0.1435	0.0819
	0.1165	0.0475	0.1396	0.0806
	0.1114		**0.1340**	**0.0735**
	0.1092		**0.1307**	**0.0693**
	0.1012		**0.1245**	

* Average rate.

A slow intravenous infusion of adrenalin (1/1,000,000 in normal saline) after the vagus endings had been paralysed with atropine, caused acceleration of the spontaneous rhythm and shortening of the refractory period. Six such observations were made. The refractory period showed an average reduction of 40 per cent as compared with the control measurements but was never shortened to the extent



TEXT-FIG. 1. R-S intervals, at which auricular fibrillation followed a single stimulus, compared with the control refractory period and that under vagal stimulation.

produced by vagus stimulation (See Table III). In Table IV are shown the alterations in the refractory period in a single auricle produced successively by vagal stimulation, by atropine and by adrenalin.

It was repeatedly observed that a stimulus introduced during vagal stimulation, shortly after the end of the refractory period, was followed not by a single response, but by auricular fibrillation. This was not encountered in the control observations before stimulation of the vagus or following atropine or adrenalin.

Such results have been previously reported by DeBoer (6) and others in hearts deprived of their blood supply or poisoned with various drugs. Lewis, Drury and Bulger (1) have called attention to the occasional occurrence of such a disorder of rhythm during refractory period determinations while the vagus is being stimulated. The development of this irregularity in our series of experiments following a stimulus applied well out on the auricular appendix is of particular interest. The fact that there is no measurable gap between the stimulus and the beginning of the re-entrant rhythm (as shown in Fig. 2) indicates, we believe, that it rises at that point and not in a ring of muscle at the base of the auricle.

Moreover this rhythm consistently followed stimuli which, in the absence of vagus stimulation would have fallen within the refractory period, Text- fig. 1. Due, however, to the shortening of the refractory period consequent upon stimulation of the vagus, they fell upon tissue which was excitable but in which conductivity had, presumably, not yet returned to normal. Hence as long as vagal stimulation was continued there existed in the auricular musculature conditions favorable to a re-entrant rhythm. The fact that, in the normal heart under conditions of increased vagal tone, an extrasystole occurring early in diastole may set up a re-entrant rhythm suggests to us a possible explanation, in some instances at least, for the genesis of auricular fibrillation.

SUMMARY

1. A method is described for determining the refractory period of the dog's auricle during the normal sinus rhythm. The advantages of the method are:

(a) The total stimulating effects of repeated induction shocks are avoided.

(b) The action current is recorded from a point one millimeter or less from the point of stimulation.

(c) Alterations in the spontaneous rate of the auricle do not interfere with the accurate determination of the refractory period.

2. The values obtained for the normal refractory period and the changes produced by atropine and by stimulation of the vagus agree closely with those of previous observers.

3. The automatic features of the method make possible the determination of the refractory period under adrenalin. This drug brings about a distinct shortening of the refractory period but less than that produced by stimulation of the vagus.

4. During vagal stimulation a single induction shock, introduced soon after the end of the refractory period, frequently produces auricular fibrillation. The cause of this irregularity is discussed and its relation to clinical auricular fibrillation is suggested.

APPENDIX

Amplifier and Timing Device

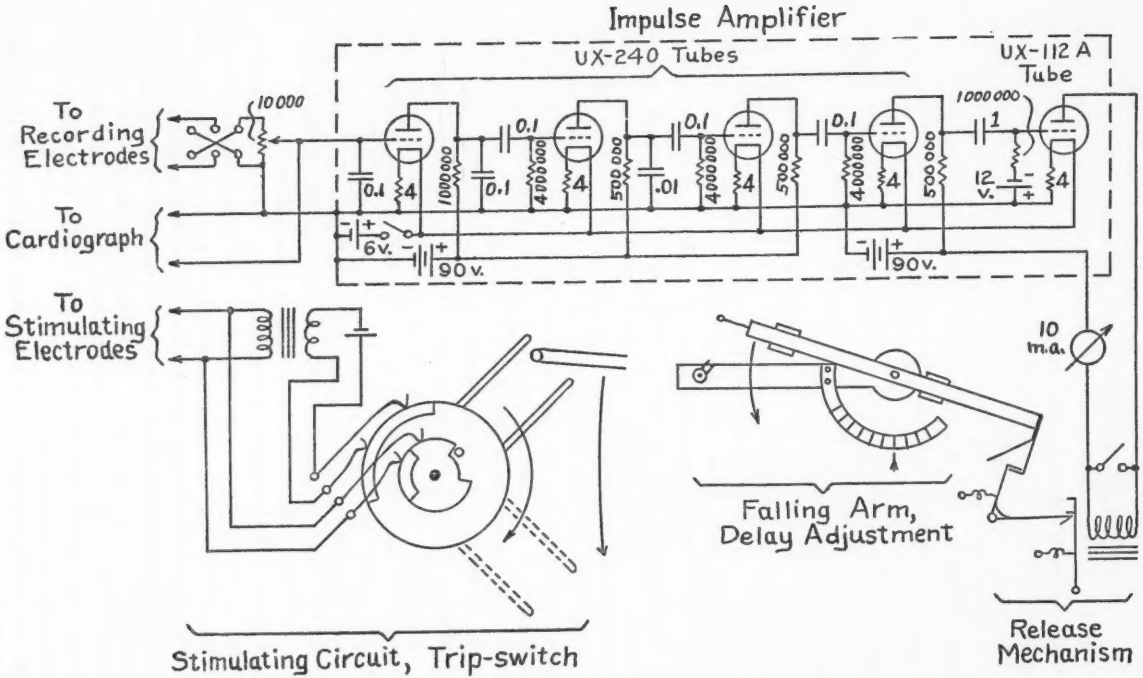
By HAROLD A. WHEELER, Ph.D.

The complete apparatus comprises a five-stage resistance-coupled vacuum-tube amplifier, greatly amplifying the cardiac action-currents, together with a magnetically-actuated mechanical system automatically introducing a stimulating induction shock. The arrangement of the apparatus is illustrated schematically in Text-fig. 2. The resistance values are given in ohms, the capacitance values in microfarads. A six-volt storage battery and two 90-volt dry batteries supply the necessary power. The recording electrodes are connected to the electrocardiograph through an intermediate 10,000-ohm resistor. Independently of the amplifier, the action-current deflection on the galvanometer is adjusted to a value between one-half and two millivolts, the apparatus being designed to operate on this terminal voltage of the galvanometer without further adjustment. The galvanometer string is at no time exposed to the output of the amplifier.

The apparatus performs two functions at the same time; it amplifies the action current to a value of more than 20 volts, and stores, and slowly discharges, energy so that the amplified voltage has a duration of approximately 1/10 second as compared with the 1/50 second duration of the unamplified action current. The amplification of the potential difference applied is thus about 200,000 times, and represents about 10,000,000 times the energy of the action currrent.

The amplified current flows through a 10-milliampere meter and a 10,000-ohm magnet. During adjustment for operation, a switch across the magnet is closed and the magnet is not actuated. The amplifier responds only to a positive potential applied to the first amplifier tube; the reversing switch in the recording electrode circuit must, therefore, be adjusted by trial to give the sharpest deflection of the meter pointer.

The mechanical system is activated upon opening the magnet switch. Each amplified action-current impulse thereafter instantly moves the magnet armature. This armature carries an escapement such that the second impulse releases a hook, which, in turn, sets free a falling arm. The falling arm operates a trip-switch,



HEART AMPLIFIER AND ACCESSORIES FOR DETERMINATION OF REFRACTORY PERIOD. H.A.W. 4-22-29

TEXT-FIG. 2

opening the primary circuit of an induction coil. The stimulating electrodes thereupon receive a shock from the secondary circuit of the induction coil. The interval between the second action-current impulse and the induction shock can be adjusted to any value between 1/20 and 1 second. The speed of the falling arm is determined by the position of two balancing weights. The angular position of the trip-switch is also adjustable so that the switch can be operated at any time during the period of the falling arm. For the present experiments, a calibration curve was prepared showing the interval at all angular positions of the trip-switch.

The instrument is designed to actuate the magnet armature in response to an action-current impulse of 1/2 millivolt for 1/50 second. The grid condensers and grid resistors thus give the entire amplifier a discharging time constant of about 1/10 second, which is sufficiently short to restore the amplifier to normal very quickly after a short impulse of several millivolts.

The condensers in parallel with the first two plate circuits are proportioned to give the amplifier a charging-time-constant of about 1/50 second. This is sufficiently short to respond quickly but sufficiently long to prevent disturb, ances in the amplifier at audio or radio frequencies. These parallel condensers also serve to increase the duration of the amplified impulse to about 1/10 second-giving the magnet ample time to respond and preventing a quick rebound of its armature. A 90-volt battery common to all five plate circuits would give feedback-coupling from the fifth to the first plate circuits with resulting low frequency oscillations; therefore two separate 90 volt batteries are necessary. With a normal grid bias on the last tube, the magnet may be actuated by random fluctuations in the plate current of the first tube ("flicker effect") amplified to a high degree. This grid bias is, therefore, operated at a high negative value, giving a normal plate current of about 1/2 milliampere. The magnet armature is adjusted to respond only to 2 milliamperes or more, so that it is not actuated by fluctuations. Some difficulty may be experienced with radio-frequency disturbances picked up by the wires connected to the first grid. This is prevented by the condenser shunting the first grid circuit, and by enclosing the entire amplifier with its batteries in a galvanized-iron box. This condenser, and the filament circuit of the first tube, are connected to the box by a short wire. Mechanical agitation has no effect upon the amplifier.

The release mechanism comprises the magnet with its armature, escapement, and trip-switch, shown schematically in Text-fig. 2. The magnet has a laminated "E" core. The middle leg of the core has a cross section of one square inch and carries a 10,000 ohm coil of 40 gauge copper wire. The armature is made of 1/32 inch iron sufficiently large to cover the three poles and is supported at its base on a horizontal pin parallel to the line of poles. It carries an escapement which, on the second impulse, releases a light hook. This release on the second impulse is important because it avoids the confusion which might occur if the magnet switch were opened during the first impulse.

The falling arm is of light construction, one foot long, and pivoted at the center. It carries two weights, whose positions are adjustable by a threaded rod along the

length of the arm. A shock-absorber catches the arm after a 90 degree fall from its initial position. The arm, when released from the hook by the magnet, engages the trip-switch. The angular position of this switch is adjustable and is indicated on a circular scale.

The trip-switch details are shown schematically on an enlarged scale. It is made up of two insulated cylinders carrying metal contact segments. The primary contactor is engaged directly by the falling arm. The secondary contactor floats on the same pin, but is engaged by a pin on the former, and moves only during the latter part of the motion of the primary contactor. When the arm is raised the primary circuit of the induction coil is first closed and the secondary shock absorbed, then the secondary circuit is opened ready for the experiment. When the arm falls, the primary circuit is first opened, producing an induction shock at the stimulating electrodes, and the secondary coil is shunted.

Thus all mechanical operations are performed by merely raising the falling arm. The trip switch is set and the induction-coil-battery circuit closed. The hook is pushed back and automatically sets the escapement on the armature. Observations can thus be made in rapid succession.

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EXPLANATION OF PLATE 8

FIG. 1. Curves recorded from the point of stimulation; (a) during control observation, and (b) during vagal stimulation. The stimulus appears as a thickening on the rising limb of the wave.

FIG. 2. Fibrillation of the auricles following a single induction shock during stimulation of the vagus. Stimulation begun at A and ended at B.

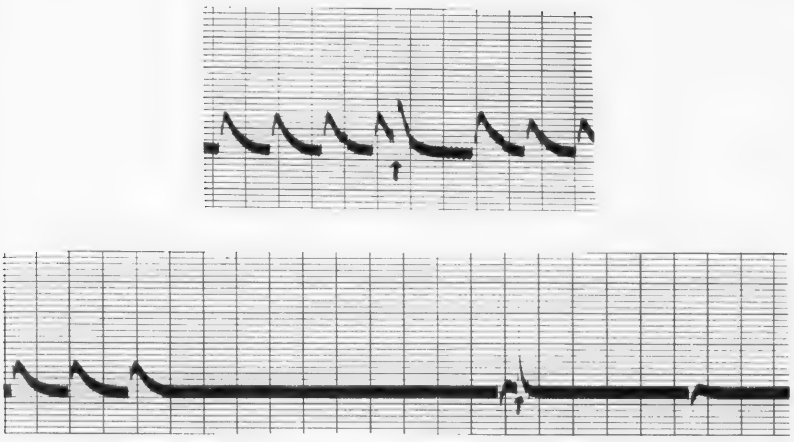

FIG. 1

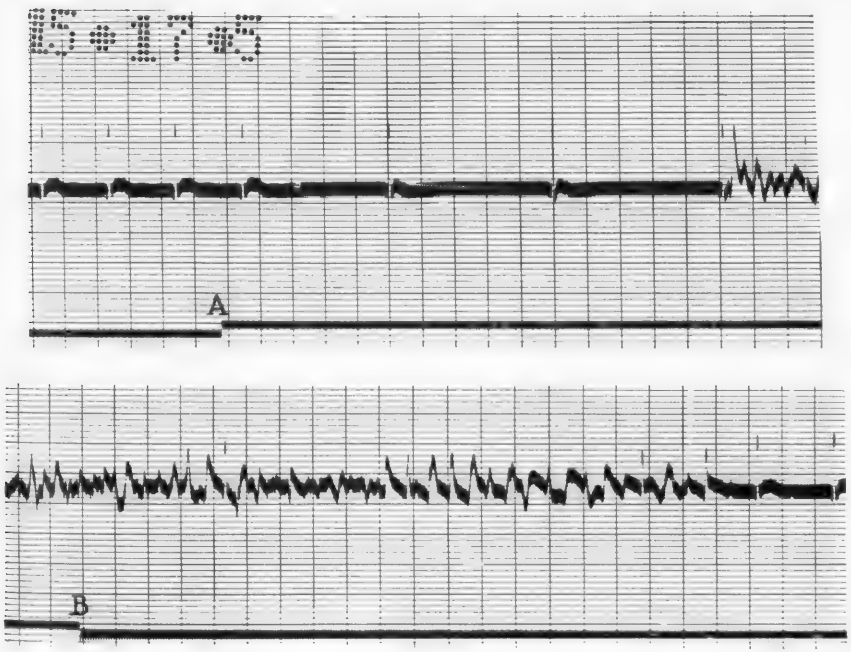

FIG. 2

(Andrus and Carter: Refractory period of normal dog's auricle)

STUDIES ON VITAL STAINING

I. Some Problems in Colorimetry. The Quantitative Analysis of Mixtures of Colored Substances in Solution

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INTRODUCTION

In the course of some experiments on vital staining a need arose for a method for quantitative analysis of various dyestuffs when in solution along with other colored substances which would interfere with measurement in any of the ordinary standard colorimeters. This problem was finally solved by use of the spectrophotometer. With this instrument one may measure the amount of light absorbed by the solution in any portion of the spectrum. A blue dye transmits blue light but absorbs strongly in the red, and by measuring the amount of this absorption in the red end of the spectrum one may calculate how much of the dye is present in the solution. Conversely, a red dye transmits red light but interferes with the passage of blue and green light, so that the dye concentration may be determined by ascertaining to what extent blue light is absorbed. In case both dyes are present simultaneously each may be determined separately by taking measurements in the two portions of the spectrum where each has a more or less specific effect. It is true that the absorption is not entirely limited to one particular region of the spectrum in the case of either dye, but in suitable cases the correction is small and can be applied in the way outlined below. Other workers (1) have considered this problem of color analysis, but from a somewhat different point of view. It is the purpose of the present paper to describe the method for the analysis of colored mixtures in general terms and to give simple equations which are applicable to the problem studied in the papers to follow. These same equations and the same method in general should be

applicable to a wide variety of problems. Spectrophotometric analysis of the sort here discussed has not received the attention which it deserves from biologists. The quantitative analysis of such mixed colored solutions should find many applications in biology. Dye solutions may be studied quantitatively, even though hemoglobin, bile pigments or urinary pigments may be present as contaminations—a condition often present in biological studies. Dyes excreted into the urine can be measured despite the presence of urinary pigments or even blood. Quantitative analyses by means of color reactions are often unsuccessful on account of extraneous pigments or side reactions. In such cases spectrophotometric analysis might be used to rule out the interfering effect of the foreign colored substance.

The method just outlined will be illustrated by the use of two dyes, brilliant vital red and Niagara sky blue, for they are the ones used in the work to be reported later, but the method of analysis of colored mixtures is applicable to a wide variety of substances, the chief condition being that the two substances mixed must be of different colors. Certain limitations of the method will be discussed more in detail later.

The Analysis of Simple Colored Solutions

There are several types of spectrophotometers which differ in various details. In all types a beam of white light is broken up by the instrument into a continuous spectrum of violet, blue, green, orange and red. If a glass cell of known thickness containing the unknown colored solution is placed in the beam of light, certain portions of the spectrum will be darkened more than others. By means of a slit in the eyepiece the view may be limited to any particular portion of the spectrum and the amount of dimming can be measured by means which vary with different types of spectrophotometer.

For ideal solutions the Lambert-Beer law states that

$$i_1 = i_0 \cdot 10^{-ECh} \tag{1}$$

or,

$$\frac{i_1}{i_0} = 10^{-ECh} = T \tag{2}$$

where i_1 is the intensity of light of any given wave length after the beam has traversed the colored solution, i_0 is the intensity before traversing the solution. C is the concentration of the latter (here expressed in terms of mg. per liter), h is the thickness of the layer of dye solution (expressed in cm.) and E is a constant which

varies with the nature of the colored substance and with the portion of the spectrum examined. The ratio of i_1 and i_0 represents the fraction of the light transmitted and is independent of variations in intensity of the light source. In some types of instrument this fractional transmission, T, can be read off directly on the scale of the instrument or can be computed by relatively simple calculation.

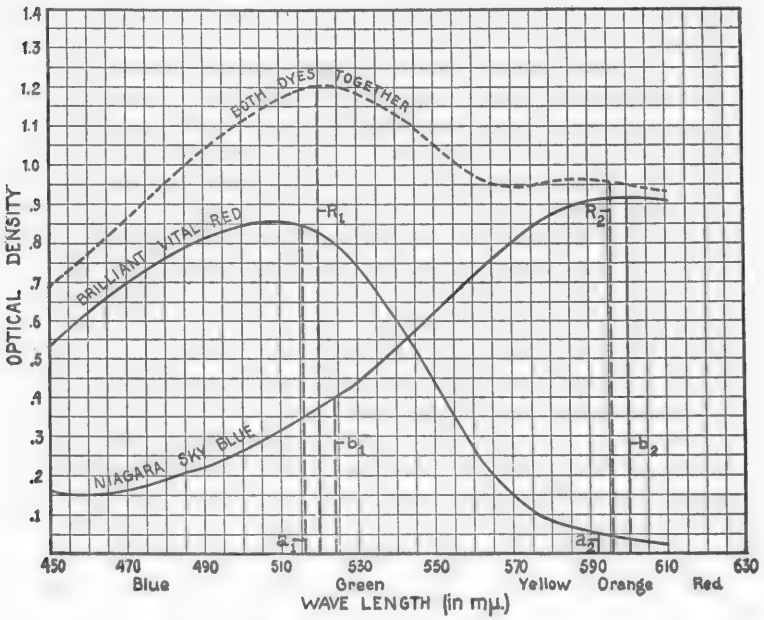

CHART 1A. Absorption curves calculated for 0.002 per cent solutions. Layer = 1 cm. thick.

Obviously our interest centers in the values of E, C and h. These factors may be grouped together by letting

(3) $$D = ECh$$

Then,

(4) $$\frac{i_1}{i_0} = T = 10^{-D}$$

D, the "optical density" of the solution may be looked upon as the obstructive power of the dye solution and is proportional to the concentration and to the

thickness of the layer. Its value varies in different parts of the spectrum depending upon the particular kind of light absorbed by that particular substance. In some types of instrument the calibration is such that D can be read directly on the scale of the photometer. In other instruments D must be calculated from the observed value of T. This calculation is very simple, since from equation 4 it is obvious that $D = -\log T$. In the Bausch and Lomb instrument used by us a control beam of light enters the instrument alongside the first beam and the two are matched by means of Nichol prisms. One glass cell containing the colored solution is placed in one of these beams of light. The other beam traverses a control cup filled with water. One should adopt the practice of reversing the cups and taking a second reading, thus avoiding errors arising from polarization and from slight maladjustment of the instrument. The readings of the Nichol prisms are shown in degrees. For purposes of precision the colored solution should be so diluted that the first reading lies between 10° and 25°. On reversing the cups a large angular reading is obtained (between 65° and 80°). From these two readings one may calculate the optical density from the formula,

$$D = \log \cot \text{small angle} + \log \tan \text{large angle}.$$

Having determined the value of D one may calculate the concentration of the colored substance by means of formula 3. This latter step necessitates knowledge of the value of E for that particular substance in that portion of the spectrum, but this value is readily ascertained by making a preliminary analysis of the colored substance in pure solution. In Chart 1A are shown the absorption curves of the two dyes, brilliant vital red and Niagara sky blue. For reasons to be discussed later the dyes were made up in a mixture of saline and normal dog plasma. The different portions of the spectrum are plotted according to their wave lengths expressed in mμ (1 mμ equals 10 Ångstrom units). The power of each dye to absorb light (D, the "optical density") is shown by the ordinates. It is seen that brilliant vital red absorbs almost none of the red light, but is quite effective in absorbing blue and green light. The maximum absorption is in the vicinity of 500–520 mμ. In quantitative analysis of this dye it is obvious that the greatest precision is obtainable by basing the calculation on readings taken in the region of the maximum density. We note that at 520 the "optical density" of a layer 1 cm. thick of the 0.002 per cent solution is 0.84. If we express concentration in terms of milligrams of dye per liter of fluid we find from equation 3 that the value of E is 0.042. In case of the blue dye the maximum absorption is in the region of 600 mμ and the value of E at this particular point is 0.046. Once these values of E have been determined one may proceed to the analysis of unknown solutions of these substances, using equation 3 for purposes of computation. Using this method of color analysis one obviates the necessity of having to prepare frequent standard solutions of these substances as in ordinary colorimetric work. One avoids unnecessary labor and at the same time is assured of results which are independent of uncontrollable variations in the standard.

The Analysis of Two Admixed Colored Substances

When one desires separate quantitative analysis of two colored substances present simultaneously in solution one may use the method previously mentioned, i.e., one may measure the absorption of light in the two portions of the spectrum where each dye has its characteristic absorption band. The situation is illustrated graphically in Chart 1A by the absorption curves of Niagara sky blue and brilliant vital red. In case both dyes are present simultaneously in equal amounts the mixture becomes purple and the absorption curve is the sum of the two individual curves and is shown by the dotted line. It is with such mixtures of colors that one has to deal in experimental work and it is our problem to deduce from this summation curve just how much of each of the two dyes is present in the mixture. For purposes of making this analysis we may choose to make readings at wave length 520 and 600, for in these regions one dye shows approximately its maximum absorption while the other shows nearly the minimum. Readings taken nearer the ends of the spectrum would be somewhat more ideal theoretically, but it is difficult in these regions to match the colors accurately in the spectrophotometer.

Referring to Chart 1 A it is seen that R_1 and R_2 can be measured in the spectrophotometer. We wish to ascertain the values of a_1 and b_2 so that we may calculate from them the concentration of each dye in the mixture.

Inspection shows that

(6) $$R_1 = a_1 + b_1$$

(7) $$R_2 = a_2 + b_2$$

But the form of the absorption curve of each of the two dyes is very specific. Hence

$$\frac{a_1}{a_2} = K_a, \text{ or } a_2 = \frac{a_1}{K_a}$$

$$\frac{b_1}{b_2} = K_b, \text{ or } b_1 = K_b\, b_2$$

where K_a and K_b are constants which can be determined for each dye by study of the latter in pure solution uncontaminated by the other dye. Substituting these values in the equations above

(8) $$R_1 = a_1 + K_b\, b_2$$

(9) $$R_2 = \frac{a_1}{K_a} + b_2$$

from which it follows that

(10) $$a_1 = \frac{K_a R_1 - K_a K_b R_2}{K_a - K_b}$$

(11) $$b_2 = R_2 - \frac{a_1}{K_a}$$

These values of a_1 and b_2 are "optical densities" and by substituting their values for D in equation 3 the concentration of each dye in the mixture can be calculated.

Before undertaking the analysis of colored mixtures one should plot accurate absorption curves of pure solutions of each substance. This gives the various constants needed and at the same time allows one to proceed wisely in choosing the points in the spectrum where measurements should be made in order to give the greatest possible precision. The relative quantities of the two substances present influence the accuracy of analysis in a way which will be evident at once. In general the substance can be determined more accurately when it occurs in large amounts in the mixture. However it is possible to measure small amounts of a substance in the presence of large amounts of another colored substance provided the absorption bands of the two substances are fairly well separated. Inspection of Chart 1A shows that brilliant vital red absorbs very little light at 600. The large absorption by Niagara sky blue at this point permits very accurate analysis of the latter. On the other hand at 520 the light absorption is by no means all due to brilliant vital red; hence the amount of the latter cannot be measured so precisely as would be possible if the Niagara sky blue absorption band were more strictly localized to the red end of the spectrum.

In all spectrophotometric analysis it has been found desirable to take readings at several points in the region of the spectrum chosen for special study. On plotting these points one will discover and correct small errors due to inexact matching of the colors. It is also wise to take readings in neutral portions of the spectrum so chosen that suspected contaminating substances will be detected when the curve is plotted. In our own experiments to be reported later we

were concerned with the analysis of dyestuffs in plasma, and under these circumstances readings were taken in parts of the spectrum where hemoglobin and bilirubin would be detected if present.

It would seem hardly necessary to remind workers in this field that hydrogen ion concentration of the solvent may totally alter the form of the absorption curve of the dissolved coloring matter. In some

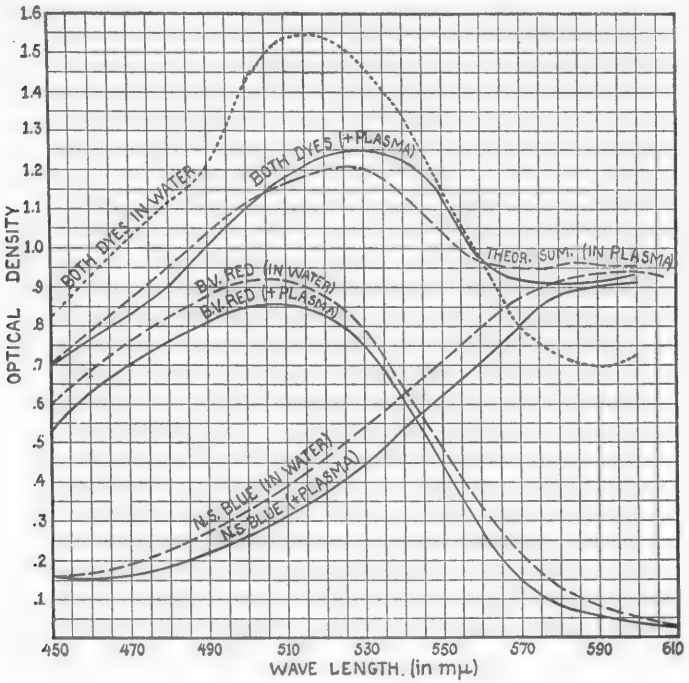

CHART 1B. Light absorption curves of brilliant vital red and Niagara sky blue (0.002 per cent solution) in water, and in plasma diluted with four parts of saline.

cases the salt concentration or protein concentration is also of great importance in its effect upon a dye. The preliminary control experiments should include a study of all such factors and their possible influence upon the color behavior of the dye. Okuneff (2), Seyderhelm and Lampe (3) and others have shown that whereas trypan blue dissolved in water is violet in color, the addition of plasma proteins

or egg-white gives the dye a more bluish color. In studying mixtures of colored substances the possibility of interaction between the two must also be kept in mind. In extreme cases precipitation may occur, but in other cases the reaction is much less evident and the only effect noted may be that the observed absorption curve of the mixture does not quite represent the summation of the absorption curves of the two components. A difficulty of this very sort arose in the case of mixtures of brilliant vital red and Niagara sky blue, and for a time it seemed as though it would be impossible to make quantitative studies of such mixtures. Curiously enough these difficulties existed only when the two dyes were brought together in aqueous solution. When a small amount of plasma protein was added to the mixture it was found that the combined absorption curve of the two dyes approached the theoretical summation curve in quite a gratifying manner. These facts are shown graphically in Chart 1B. The discrepancy from theory in pure aqueous solution is less marked in the blue than in other portions of the spectrum. In the green it amounts to about 30 per cent, and well over in the red near 590 the discrepancy is almost as great, though in the opposite direction. From the standpoint of quantitative analysis it is most fortunate that a little plasma protein will correct these abnormalities in light absorption and give values so closely approximating the theoretical summation curve shown by the interrupted line in the chart. It is rather remarkable that relatively small amounts of plasma need be added to bring about this result. Experiments have shown that one part of plasma to ten of the color mixture is adequate. The plasma of vitally stained dogs may contain large quantities of the two dyes and in such cases it may be necessary to dilute it preparatory to making spectrophotometric readings. In order to preserve an adequate concentration of the stabilizing proteins it is well to use saline mixed with normal dog plasma as a diluent. Normal plasma has a weak absorption curve of its own, but this may be ascertained in advance and proper correction made if necessary.

As yet we do not fully understand how the proteins bring about this stabilizing effect on the color absorption curve, though it is suggested that the proteins enter into some form of combination with the dyes and thus prevent or break up loose combinations which tend to form between the dyes themselves. It is noteworthy that either dye alone

in solution gives a color absorption curve which is largely independent of the presence or absence of plasma proteins. It would appear to be a combination of the dyes which is affected by the proteins.

It is clear that the effect on the dyes cannot be attributed to the buffer action of the plasma, for these particular dyes are relatively insensitive to changes in acidity unless very large amounts of acid are added. Furthermore, we can show that the two dyes made up together in selected inorganic buffer solutions show color absorption curves almost identical with those obtained in pure aqueous solution, and like the latter the deviation from the theoretical is quite marked unless plasma protein be added also.

SUMMARY

1. A spectrophotometric method is discussed which permits quantitative analysis of colored substances present in mixtures

2. Special attention is given to the analysis of mixtures of two dyes which are being used in a series of studies on vital staining.

3. It is shown that the method can be applied to quantitative analysis of mixtures of naturally occurring animal pigments or to mixtures of these with various other colored substances.

4. Certain limitations of the method and certain necessary precautions are discussed.

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STUDIES ON VITAL STAINING

II. The Removal of Brilliant Vital Red from the Blood Stream. Distribution of Dye between Blood Stream and Body Tissues

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INTRODUCTION

In recent years there has developed a voluminous literature which deals with the phagocytosis of foreign materials by various body cells. Ink, bacteria and various dyes have been introduced into the living animal until the tissues were heavily laden with these substances. There has been much discussion about the possibility that one may saturate the tissues to the point that certain of their physiological powers are destroyed, or at least impaired. References to the literature may be found in the monographs of Aschoff (1) and of Börner-Patzelt, Gödel and Standenath (2).

These foreign substances with which we are concerned are taken up and stored in granular form within a vast number of cells, some of which are endothelial in nature, while others are wandering or fixed cells of connective tissues. Details concerning the histological aspects of the problem will be given in a subsequent paper (3). The phagocytic cells scattered throughout the body have been referred to collectively as the "reticulo-endothelial system." Many and varied physiological activities have been ascribed to them. Much has been written of their activity in freeing the blood stream and the tissues generally of objectionable particulate matter, such as bacteria, dead cells and cell debris. There is much evidence to indicate an ability on their part, to transform hemoglobin to bile pigment. It has been supposed that they may play a part in fat metabolism and by some they are thought to be concerned in the elaboration of immune bodies. Still other supposed activities are discussed in the monographs cited above. Many of these theories rest on the most insecure basis. Changes in pigment metabolism, in immune body balance, etc. are often observed to follow injection of certain foreign substances such as ink or carmine. Those who think that ink or carmine paralyze or "block" the reticulo-endothelial system will attribute all of these reactions to changes in the latter, and, totally disregarding possible changes in other organs or tissues, they will find no end of "functions" of this system. Other

workers, more interested in settling the question of "blockade" than in establishing new functions of the reticulo-endothelial system are sometimes rather hasty in assuming that the system has this or that function, and from changes observed they may draw erroneous conclusions in regard to the existence of a "blockade."

Thus, each of these groups of workers assumes the point which the other tries to prove by experiment. It is easily seen that such arguments are in a circle and are utterly futile. We would suggest that much desirable information concerning "blockade" may be obtained by careful study of reactions in which the rôle of the reticulo-endothelial system cannot be questioned. In the present state of knowledge we feel that much uncertainty prevails concerning the great majority of the "functions" of these cells. We feel that more is to be gained by a study of their long-known function of phagocytosis, or "storage" of dyes or other materials.

In the case of many dyes this process can be followed with the microscope, but unfortunately the purely morphological studies are almost entirely qualitative. They furnish little information about the degree or the speed of phagocytosis. The rate at which dyes leave the blood stream gives a better quantitative measure of such activity. However, many dyes and other substances in current use disappear quite rapidly from the blood stream and in many cases the particles tend to agglutinate in capillaries even before they are taken up by phagocytes. Other substances pass out rapidly through the bile or urine or both. Obviously in none of these cases is the rate of disappearance from the blood stream a trustworthy criterion of phagocytic activity. It should be stressed that most of the substances used in the past have had these defects to a striking degree. As yet no ideal test dye has been discovered, though a few approach the ideal much more closely than the rest.

A large number of dyes has been studied by Dawson, Evans and Whipple (4) and much valuable information has been obtained concerning the rate at which they leave the circulating blood. One of these, brilliant vital red, has already received further study from this standpoint by the present author (5). The dye is relatively nontoxic. It is not readily precipitated in neutral or nearly neutral solutions by the salts common in the animal body. The elimination through the kidneys is negligible. However, the dye is largely eliminated through the bile over the course of several days, but in the meantime the tissues become deeply stained and microscopic examination shows the phagocytes everywhere to contain beautiful accumulations of the red dye. During the first 2 or 3 days following injection the amount lost through the liver accounts for distinctly less than half of the dye which leaves the blood stream. The rest is taken up by the tissues, largely within phagocytic cells.

In an earlier paper of this series (5) a study was made of the rate at which the dye leaves the blood stream, and curves were plotted to show the relations graphically. The concentration in the blood stream falls off rather rapidly during the first few hours, but much more slowly later on (see also Chart 2C of the present paper). At the end of about 48 hours considerable dye still remains in circulation and from this point on the dye is so slowly eliminated that the curve is almost horizontal. No doubt the curve would approach the horizontal still more closely were it not that a small amount of dye is continuously being lost by excretion into the bile. The tissues are now deep red and it is clear that they are not taking up dye as readily as they would do in their normal unstained condition.

It might have been anticipated that the phagocytes would ingest dye quite rapidly at first when the plasma and lymph about them contain such large amounts of dye, and this is quite in accord with general experience, but there seems to be little recognition of the fact that the phagocytic activity slows up long before all of the dye has been ingested. With larger doses, phagocytosis also slows up but leaving still larger quantities of dye in circulation than before, and along with this one notes that the phagocytes contain more dye. To explain these facts it was suggested that an equilibrium is established between dye in cells and dye in the fluids, and that the diminution in phagocytic activity is in part merely apparent, for renewed activity is observed if we disturb the equilibrium by injecting more dye. Very clearly this concept has important bearings on the question of "blockade" of the body phagocytes and it is of great importance to know whether these dye-laden cells will remove newly injected dye as rapidly as though they themselves were free of dye. Our initial observations about to be presented seemed to indicate that some impairment really exists, for we noted that after a course of vital staining new offerings of dye leave the blood stream less rapidly than normal. We found also that large doses of dye do not leave the plasma as rapidly in proportion as do smaller ones. But certain observations on bile fistula dogs placed these experiments in a new light, and it now seems that the retention of dye in the plasma is associated with inefficient liver elimination which is seen only when large amounts of dye are given. We can offer no evidence to indicate that the liver

tissue is actually injured by the dye. It may be entirely within the realm of normal physiology that 25 per cent of a small dose of dye passes into the bile in 24 hours whereas only about 10 per cent of a large dose will be excreted in such a period. The greater retention of dye within the body may well explain the retention of dye within the plasma, without our having to assume defective phagocytic activity on the part of the tissues. We wish to stress these observations and this interpretation in order to show the danger of assuming a blockade of phagocytes merely because dye leaves the blood stream unduly slowly. This view will be given further emphasis in a subsequent paper (6) where it will be shown that a small injection of India ink will inhibit almost completely for some days the excretion of brilliant vital red by the liver. The great retention of dye in the body during this period seems to be quite sufficient to account for the unduly great dye concentration in the plasma, and there is reason to believe that part of the retained dye has found its way into the tissues and that there is increased coloration there as well as in the plasma. There is little need to assume that the ink had inhibited the activity of the phagocytic system. These observations are in accord with our present contention that large amounts of brilliant vital red within the tissues need not inhibit the entrance into the phagocytic cells of newly injected dye. The disproportion in liver excretion seems to account for all of the findings which might be taken to indicate such impairment in phagocytosis.

Methods

Healthy adult dogs maintained on a mixed diet were used in all experiments. To allow accurate colorimetric measurement of dye in the plasma, feeding hours were so arranged that the plasma would show no lipemia during the morning hours when samples of blood were commonly taken for analysis. The dogs had free access to water at all times. The dye used was brilliant vital red which was obtained from the National Aniline and Chemical Company. Dye from the same bottle was used throughout the course of the experiments. At intervals a filtered two per cent aqueous solution was made up. In each case the color intensity of this stock solution was checked spectrophotometrically just after preparation and also at intervals later on, so that constancy in preparation and absence of fading could be demonstrated.

Blood for analysis was collected in graduated 15 cc. centrifuge tubes containing 2 cc. of a 1.6 per cent sodium oxalate solution to prevent clotting. The tubes

were centrifuged and the amount of plasma read off on the tube, thus making it possible to correct for the dilution occasioned by the 2 cc. of oxalate solution previously added. The amount of dye in the plasma was determined by means of the spectrophotometer. Details of this method of analysis are given in a previous article (7). In all cases the concentration of dye in the plasma is expressed in terms of milligrams of dye per liter of plasma.

EXPERIMENTAL

If brilliant vital red be injected into the blood stream, the blood plasma taken several minutes later will be found to be bright red. If the amount of dye in such plasma be measured by colorimetric means we find that the concentration is almost exactly proportional to the amount of dye injected. With given dosage, proportional to body weight, this original dye concentration varies somewhat in different dogs depending on variations in blood volume. If such a standard dose be adopted as routine, it is found that this original high dye concentration is not maintained, but the concentration falls off, so that only 15–25 per cent of the dye remains 24 hours later. From this point on the fall is much more gradual, and we believe that this slowing up is related to the fact that the tissues are becoming stained with dye and on this account do not take up dye from the fluids as readily as normal. To be sure, the amount of dye now in circulation is not large, but we know that normal unstained tissues will attract such small amounts of dye quite readily, as one can demonstrate by injecting small doses into normal unstained dogs. This delay on the part of the stained tissues has been considered briefly in a previous publication (5). It was suggested that dye passes from plasma to tissues until an equilibrium partition is approached. We may believe that the amount of dye in the blood stream would finally become quite stationary were it not that a small amount of dye is constantly spilling over into the bile and is lost from the body.

Dog 24–74. Shepherd, 30 kgm.

May 23, 1927. Thirty cubic centimeters of a 2 per cent solution of brilliant vital red injected intravenously. Five minutes after the injection the concentration of dye in the plasma was 440 mg. per liter plasma; after 1 hr., 370; 6 hrs., 250; 24 hrs., 130; 48 hrs., 42; 72 hrs., 15.

May 26 to June 2 inclusive, daily injections of 30 cc. of a 2 per cent solution of the same dye. Five minutes after the last injection the plasma contained 670

mg. dye per liter plasma; 6 hrs. later, 525; 24 hrs., 335; 48 hrs., 190. Immediately following the collection of this last sample 30 cc. more of the dye were injected. Five minutes after this injection the plasma contained 540 mg. dye per liter plasma. After 6 hrs., 370; 24 hrs., 270; 48 hrs., 160; 72 hrs., 100.

Several months later almost all of the dye had been eliminated from the body and the tissues were again in a normal condition, suitable for further experiments.

August 3, 1927. Thirty cubic centimeters of a 2 per cent solution of the dye were injected intravenously. Five minutes later the plasma contained 415 mg. dye per liter plasma. One hour later, 335 mg.; 6 hrs., 245; 24 hrs., 84; 48 hrs., 30; 72 hrs., 19.

August 6 to 13 inclusive, daily injections of 30 cc. of the 2 per cent dye. Five minutes after this injection the plasma contained 740 mg. dye per liter plasma; 6 hrs. later, 550; 24 hrs., 360; 48 hrs., 185; 72 hrs., 102; 96 hrs., 67; 120 hrs., 45.

Dog 24–96. Shepherd, 18 kg.

May 24, 1927. Twenty cubic centimeters of a 2 per cent solution of brilliant vital red injected intravenously. Five minutes after the injection the concentration of dye in the plasma was 470 mg. dye per liter plasma; after 1 hour, 410; 6 hrs., 310; 24 hrs., 160; 48 hrs., 103; 72 hrs., 62.

May 27 to June 3 inclusive, daily injections of 20 cc. of the 2 per cent dye solution. Five minutes after the last injection the plasma contained 990 mg. dye per liter plasma; after 6 hrs., 735; 24 hrs., 520; 48 hrs., 420; 72 hrs., 300; 96 hrs., 205; 120 hrs., 180.

After several months had elapsed and the tissues had rid themselves of almost all of the dye the animal was used for the following experiments.

August 6, 1927. Twenty cubic centimeters of the 2 per cent dye solution were injected intravenously. Five minutes later the plasma contained 480 mg. dye per liter; after 1 hr., 430 mg. dye; 7 hrs., 295; 24 hrs., 138; 48 hrs., 76; 72 hrs., 48.

August 9 to 16 inclusive, daily injections of 20 cc. of 2 per cent dye solution. Five minutes after the last injection the plasma contained 830 mg. dye per liter plasma; after 6 hrs., 740 mg.; 24 hrs., 575; 48 hrs., 320. Immediately following the collection of this last sample 20 cc. more of the dye solution were injected. Five minutes after this injection the plasma contained 695 mg. dye per liter plasma; after 6 hrs., 625 mg.; 24 hrs., 390; 48 hrs., 250; 72 hrs., 210.

Dog 25–16. Airedale, 23 kgm.

May 22, 1927. Twenty cubic centimeters of a 2 per cent solution of brilliant vital red injected intravenously. Five minutes after the injection the concentration of dye in the plasma was 400 mg. per liter plasma; after 1 hr., 325 mg.; 6 hrs., 240; 24 hrs., 105; 48 hrs., 54; 72 hrs., 31.

May 25 to June 1 inclusive, daily injections of 20 cc. of a 2 per cent solution of the same dye. Five minutes after the last injection the plasma contained 790 mg. dye per liter plasma; 6 hrs. later 670; 24 hrs., 450; 48 hrs., 325. Immediately following the collection of this last sample 20 cc. more of the dye were injected. Five minutes after this injection the plasma contained 660 mg. dye per liter plasma. After 6 hrs., 530; 24 hrs., 375; 48 hrs., 280; 72 hrs., 200.

Several months were now allowed to elapse so that the dye might be eliminated from the body and the tissues become pale once more in preparation for the experiments to follow.

August 4, 1927. Twenty cubic centimeters of a 2 per cent solution of the dye were injected intravenously. Five minutes later the plasma contained 340 mg. dye per liter; 1 hr. later, 320; 6 hrs., 205; 24 hrs., 95; 48 hrs., 71; 72 hrs., 28.

August 7 to 14 inclusive, daily injections of 20 cc. of the 2 per cent dye. Five minutes after this injection the plasma contained 700 mg. dye per liter; 6 hrs. later, 510; 24 hrs., 355; 48 hrs., 225; 72 hrs., 155; 96 hrs., 123; 120 hrs., 100.

Dog 25–29. Collie, 28 kg.

May 21, 1927. Twenty-four cubic centimeters of a 2 per cent solution of brilliant vital red injected intravenously. Five minutes after the injection the con-

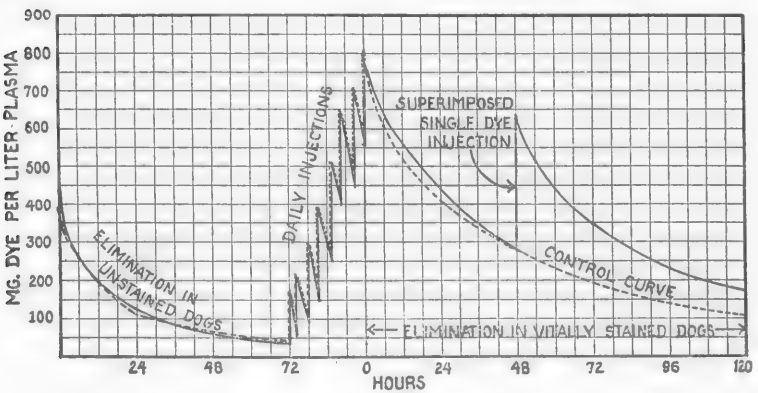

CHART 2A. Concentration of brilliant vital red in plasma (average of four experiments).

centration of dye in the plasma was 360 mg. per liter; after 1 hr., 340; 6 hrs., 280; 24 hrs., 114; 48 hrs., 59; 72 hrs., 43.

May 24 to 31 inclusive, daily injections of 24 cc. of a 2 per cent solution of the same dye. Five minutes after the last injection the plasma contained 806 mg. dye per liter plasma; 6 hrs. later, 625 mg.; 24 hrs., 405; 48 hrs., 315; 72 hrs., 230; 96 hrs., 170; 120 hrs., 110.

After several months had elapsed and the tissues had rid themselves of almost all of the dye the animal was used for the following set of observations.

August 5, 1927. Twenty-four cubic centimeters of a 2 per cent solution of the dye were injected intravenously. Five minutes later the plasma contained 390 mg. dye per liter; after 1 hr., 370 mg.; 6 hrs., 240; 24 hrs., 114; 48 hrs., 62; 72 hrs., 49.

August 8 to 15 inclusive, daily injections of 24 cc. of the 2 per cent dye solution. Five minutes after the last injection the plasma contained 810 mg. dye per liter plasma; after 6 hrs., 605 mg.; 24 hrs., 425; 48 hrs., 280. Immediately following the collection of this last sample 24 cc. more of the dye were injected. Five minutes after this injection the plasma contained 620 mg. dye per liter plasma; after 6 hrs., 540 mg.; 24 hrs., 350; 48 hrs., 220; 72 hrs., 180.

In order to learn more about the behavior of dye-stained tissues we have made daily dye injections into a number of dogs until their tissues were quite heavily stained. Under these circumstances the

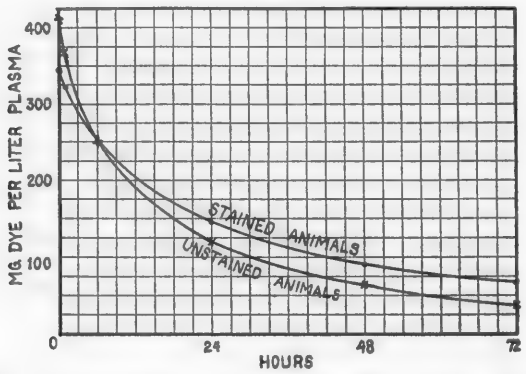

CHART 2B. Concentration of brilliant vital red in plasma (average of four experiments).

amount of dye circulating in the plasma may be very large. Even for 48 hours after the last injection rather large amounts of dye are still present in the plasma, but the rate at which the dye is leaving the circulating blood is very much reduced. The tissues are now deep red in color—a result of the dye granules present in the phagocytes. We might expect that these cells, partially filled with dye, would refuse further offerings, or at least would show a sluggish response. That they do not refuse altogether is most readily demonstrated by the injection of a test dose of dye. We are presenting 4 experiments which show this quite convincingly. The protocols are presented individually and Chart 2 A shows the average of all four dogs (Dogs 24–74, 24–96, 25–16, and 25–29).

The jagged peaks in the mid-portion of the chart illustrate schematically the rise in dye in the plasma accompanying the 8 daily intravenous injections of dye. At the end of this period the plasma was found to contain approximately 800 mg. dye per liter. The dotted control curve proceeding from this point shows that the dye leaves the plasma rather rapidly at first, but it is noted that 48 hours later the plasma still contains nearly 300 mg. dye per liter, and there are 100 mg. per liter at the end of 120 hours. It is seen that the curve is rapidly approaching a plateau. We believe that the elimination of dye by the liver is largely responsible for the fall noted in the last few hours of this curve. The tissues are still deep red. This is well seen in the skin and mucous membranes.

If several months are allowed to elapse the tissues will become almost entirely free of dye and the experiment may be repeated with almost identical results. Such a duplication is shown by the continuous line in this same chart. As in the control experiments it will be noted that following 8 daily injections the plasma again contained about 800 mg. of dye per liter, and that from this point on the two curves are very similar, and as before described the curve begins to flatten out decidedly after about 48 hours, and as before we also note that the tissues are deep red.

At this time a large test dose of dye was injected in order to ascertain how readily these dye-laden tissues would dispose of additional amounts of dye. As a result of this superimposed injection the dye concentration in the plasma promptly rose from about 300 mg. per liter to somewhat over 600. The curve from this point on shows rather rapid elimination at first, proving beyond doubt that the phagocytic activity was far from being abolished by the presence of the dye which these cells contained. We note, however, that the initial rapid loss of dye from the plasma is not maintained and the curve ultimately approaches a plateau which is nearly twice as high as that of the control curve shown by the dotted line.

We may look upon the space between these two curves as representing the reaction of the dye-stained tissues to this final superimposed dye injection. This difference may be measured at a number of points on the curves and the differences plotted separately. It is of great interest to compare the curve so obtained with a curve show-

ing the results in unstained dogs, and for purposes of comparison we have determined the normal elimination rate for these same dogs. The results are shown in the first portion of the chart, before the tissues had been stained by the daily injections. This normal curve is plotted separately in Chart 2 B and alongside this curve is the other which shows the arithmetical differences between the two curves just discussed. It will be observed that the slopes of the curves are decidedly different, and it is seen that when the dogs are vitally stained the dye leaves the plasma more slowly than normal, evidence

TABLE 21

	Dye per 1000 cc. plasma						
	5 min.	1 hr.	6 hrs.	24 hrs.	48 hrs.	72 hrs.	96 hrs.
	mg.	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*
Large dose (40 mg. per kg.)							
Dog 28–161	1020	885	700	410	225	147	71
Dog 28–158	850	710	570	325	152	94	42
Dog 28–146	865	745	530	305	165	84	52
Dog 28–107	1150	880	785	440	220	89	45
Av. all dogs	971	805	646	370	190	101	53
Small dose (8 mg. per kg.)							
Dog 28–161	157	150	118	56	26	8	
Dog 28–158	178	157	110	38	18	8	
Dog 28–146	154	133	95	37	21	7	
Dog 28–107	205	181	128	75	44	6	
Av. all dogs	174	155	113	47	27	7	

which might be accepted as proof that the tissues were becoming saturated with dye were it not that we know that liver excretion may greatly alter the amount of dye remaining in the body and hence in the plasma also. Even this factor of liver excretion could be disregarded if it were known that the amount of dye excreted into the bile were always directly proportional to the amount present in the plasma. Recent work in this laboratory shows quite conclusively that this is not the case. Part of this data will be presented in a later paper of this series (6) and part is left for future publication. We can show (6) that a small injection of India ink may have little or no effect on the volume of bile excreted, nor on the daily output

of bile salts and bile pigments, and despite this the liver may be almost completely unable to excrete brilliant vital red for 48 hours or longer. We know little of other conditions which may affect one function of the liver so specifically to the apparent exclusion of certain others. It is hoped that experiments now in progress will shed light on a field so little understood. Our recent studies also show that increasing

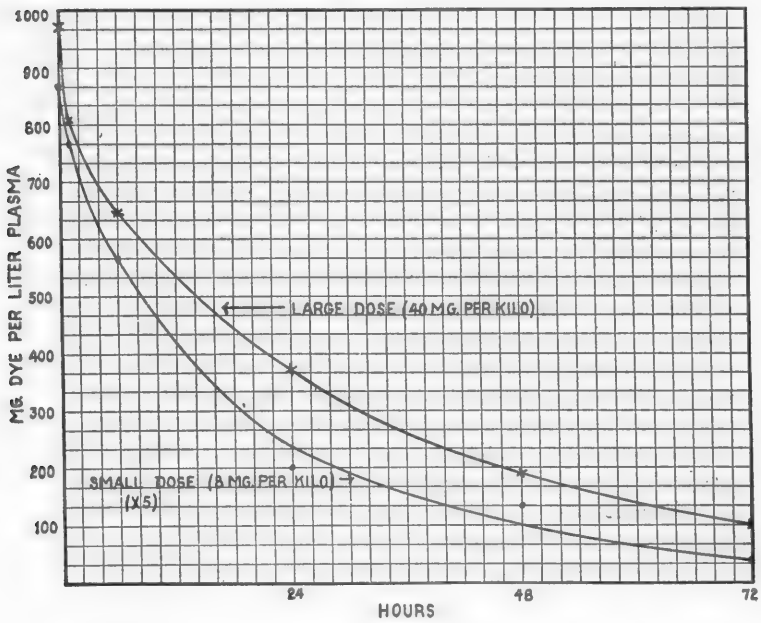

CHART 2C. Varying dose of brilliant vital red (average of four dogs—see Table 21).

the dye concentration in the plasma 5 fold does not increase liver excretion by a like amount. In fact the excretion is little more than doubled under these conditions. From this it must be clear that with large dye doses the dye is retained in the body relatively much more effectively. Such conditions must prevail in vitally stained animals where there is much dye in the plasma as well as in the tissues. The inefficient liver excretion under these conditions must go far to account

for the longer retention in the plasma of test doses injected to study the disappearance rate. In fact we believe that much of the retained dye does pass into the tissues, giving them as well as the plasma a much more intensive coloration.

Experiments like these to compare large and small injections have also been carried out on normal dogs without the bile fistula operation, and in these experiments we have made it a special point to determine the rate at which the dye leaves the blood stream. The results with 4 such dogs are given in Table 21. All gave essentially similar results, the averages of which are shown graphically in Chart 2C. With a large intravenous injection (40 mg. per kilo body weight) we observe that the plasma taken 5 minutes later is deep red in color. It contains nearly 1000 mg. dye per liter. Twenty-four hours later there are still nearly 400 mg. per liter and at the end of 72 hours about 100 mg. still remain in circulation. This curve is to be contrasted with one with a small dye injection made a number of weeks previously into these same dogs. At that time only 8 mg. per kilo had been injected. This dosage is only one-fifth that given in the later experiment, and in order to simplify comparison of the curves we have multiplied the values observed by 5, and in Chart 2C we have plotted the results alongside the curve resulting from the large injection. The curves thus brought to the same scale are quite similar for the first hour or so, but one observes that after that they tend to separate rather rapidly and at the end of the experiment one curve is almost twice as high as the other. It is noted that the marked lag is shown in the curve resulting from the large injection. It is quite obvious that dye does not leave the circulation five times as rapidly as with a dose one-fifth as large. We feel that the phagocytic activity may not be quite proportional to the dosage of dye and that with large dosage the undue delay in elimination may be due in part to this, but we are convinced that much if not all of the discrepancy between the two curves should be attributed to the fact that the liver does not excrete the large dose five times as rapidly as the smaller one.

DISCUSSION

The experiments just presented have shown that following multiple daily injections or, indeed, after a single large injection, much of the

dye remains in circulation for many days. The rapid elimination during the first hours stands quite in contrast to the slower elimination later on. After several days the amount in the plasma remains almost constant. A state has been reached such that the tissues take up little dye unless the concentration in the plasma is artificially raised by further dye injection. We feel that this relative inactivity of the tissues must be taken to mean that an approximate equilibrium has been established between dye in plasma and dye in the tissues. We know that this inactivity is not due to a lowering of the plasma dye concentration below a hypothetical functional threshold value, for we have shown that with small doses the normal unstained tissues will attack such amounts of dye with great vigor. The concept of an equilibrium between dye in plasma and dye in tissues is also borne out by work still in progress which goes to show that by rapid bleeding and transfusion of vitally stained dogs one can reduce the dye concentration in the circulating plasma to a very low level, and in such cases we have observed that dye passes back from tissues to blood stream, and for a number of hours the dye concentration in the blood stream increases. We can thus demonstrate the essential features of an equilibrium reaction, namely that the progress is reversible and the tendency is to resist or compensate for a displacement produced in either direction.

Quantitative concepts of dye partition between cells and the fluids which surround them received scant attention in the older literature. So much of the work concerns the permeability of cell membranes and here the problem of equilibrium is neglected. Other workers have been more concerned with the power of cytoplasm to dissolve dyes or to unite with them in one manner or another. Certain of these workers have had the equilibrium concept clearly in mind; others have been less specific, though in certain cases the concept seems to be implied. Attempts to compile quantitative data are almost completely lacking until very recently. Within the past few years Irwin (8) has made notable contributions to the study of the distribution of certain basic dyes between plant cells and the fluids in which they are immersed. Many factors were found which influence this distribution, but she is convinced that with constant conditions the amount of dye within the cell is proportional to the concentration of dye in the sur-

rounding fluid. Collander (9) has also made studies on plants, but with acid dyes. He gives much thought to the question of permeability, and the equilibrium concept is much less fully treated than in the work of Irwin. These quantitative studies of Collander and of Irwin involve plant cells only. The diffuse coloration of the sap of plant cells may well be comparable to the diffuse staining sometimes seen in animal cells, but it is difficult to relate this process to the granular storage in macrophages of certain acid dyes such as brilliant vital red. The brilliant and deeply colored granules which form within the macrophages are almost certainly not formed from simple staining of preexisting protoplasmic granules within the cell, though we must admit that the dye may exist in union with substances not previously segregated into discrete foci. Schulemann (10) and Evans and Scott (11) have stressed the view that the behavior of this group of dyestuffs in the body may be closely akin to the process of phagocytosis of small bits of particulate matter so familiar to all. There is the added feature that during the process of granule formation the cells must take the dye from solution and build it up into small concentrated microscopic aggregates. We wish to stress our observation that even in this type of staining, so closely related to phagocytosis, we can demonstrate the existence of an equilibrium between the dye in cells and the dye in the surrounding fluids. It is perhaps less surprising that such a principle holds where the dye seems more clearly to enter into union with preexisting materials within the cells. We should be more surprised to find it in the case of acid dyes where the granules are built up *de novo* within the cells by a process of concentration and storage far more elaborate in character.

We feel that studies concerning equilibria should be extended to other types of cells and to other dyes. Such studies should illuminate the questions regarding whether in various cases the dye within the cell is merely dissolved in fluids and lipoids of the cell or whether it exists in the form of chemical or physico-chemical combination with elements present in the cell. Very possibly no single rule holds for all dyestuffs and for all cells.

SUMMARY

Brilliant vital red injected into the blood stream of dogs is slowly taken up by phagocytes in various parts of the body, but eventually

an equilibrium is established, after which the concentration as measured in the plasma remains almost constant for long intervals of time.

This equilibrium can be disturbed by injecting more dye, and in this case the phagocytes resume ingestive activity, apparently with normal or nearly normal vigor. This activity continued until a rather large part of the newly injected dye has been removed, and as the reaction again slows up we note that both plasma and tissues contain more dye than before. It is difficult to be certain that the distribution ratio of dye between plasma and tissues remains unaltered with dosage, but evidence indicates that for non toxic doses, at least, this is approximately true.

This study of this partition ratio is complicated by the fact that the liver slowly excretes dye into the bile, and this helps to reduce the amount of dye in the body. Partial correction for this factor can be made by ascertaining the dye output in bile fistula dogs. These latter studies show that dye elimination into bile is *relatively less efficient* when large doses of dye are given to the animal than with smaller dosage. This undue retention of dye in the body with large dosage helps to maintain the dye concentration in the plasma at unduly high levels. These peculiarities in liver excretion have an important bearing on liver physiology in general, and in addition they also have an important application in connection with the theory of "blockade of the reticulo-endothelial system." It is now obvious that prolonged retention of dye in the blood stream does not of itself prove that this group of phagocytic cells is "blocked" against the entrance of foreign material. Altered excretion by liver, kidney, etc. must be ruled out before we can accept such data as evidence of "blockade."

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STUDIES ON VITAL STAINING

III. The Simultaneous Ingestion of Two Dyestuffs by Phagocytes. The Question of "Blockade of the Reticulo-Endothelial System"

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INTRODUCTION

In previous articles of this series (1) it has been shown that brilliant vital red is relatively non toxic to dogs when given intravenously in rather large amounts. The dye is removed from the plasma, and prior to its gradual elimination from the body is stored in various tissues, mainly in the large groups of phagocytic cells sometimes spoken of collectively as the "reticulo-endothelial system." It was shown that the dye passes from plasma to tissues for many hours, but eventually an equilibrium point is approached, and from this time on dye leaves the circulation very slowly. This virtual cessation of phagocytic activity may appear long before all of the dye has left the circulating plasma, and this is true especially when large amounts of dye are injected. It is in these very cases that the tissue phagocytes themselves are most deeply stained. We concluded that the cessation in phagocytic activity is the result of the partial filling of these cells with dye, but it was possible to show that on injecting more dye the phagocytes will at once resume their activity, and for a time dye leaves the circulation rather rapidly. Quite evidently this injection simply disturbed the balance between dye in plasma and dye in tissues, and the balance could not be restored until part of the newly injected dye had passed from plasma to tissues. This state of balance illustrates a curious form of inactivity on the part of living cells. This inactivity is clearly associated with the presence of dye in the tissues. This is not an inhibition or "blockade" in the ordinary sense of the word, for if we

disturb the equilibrium by injecting more dye we find that the dye-laden cells show a surprising ability to take up the newly offered dye. The evidence indicates that the phagocytic response toward new dye may equal that of normal unstained tissues.

It is of great interest to investigate the reactions of these tissues toward the introduction of a second dyestuff—for analytical convenience a blue dye. We may hope to ascertain whether the presence of the red dye in the tissues will inhibit the entrance of the blue one and whether the final partition of the blue one between plasma and tissues will be influenced by the presence of the red dye in the body. Such studies involve the existence of what we might term "cross inhibition," and it is this form of inhibition to which most workers refer when they speak of "blockade." We may apply the term "specific inhibition" to the concept that tissues laden with one particular dye are so affected that they take up this particular dye not at all or with distinct reluctance. In our own studies such "specific" inhibition was clearly demonstrable only in the sense of an equilibrium between dye in tissues and in plasma. It was impossible to demonstrate "specific" inhibition in the larger sense which involves reluctance of tissues to take up subsequent offerings of this particular dye.

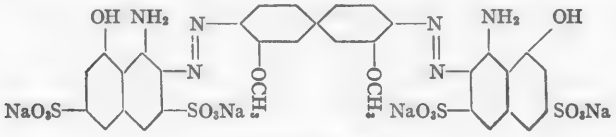
The experiments about to be reported deal with the question of "cross" inhibition, and for purposes of investigation we have injected aqueous solutions of brilliant vital red and of Niagara sky blue. These two dyes belong to the same general class. With the exception of color both have many physical properties in common. Both have certain colloidal properties. In the matter of diffusibility they are rather closely similar. The general similarity in chemical constitution is shown by the structural formulae given below. From these numerous points of similarity one might anticipate finding many points of similarity in physiological behavior also, and we need not be surprised that the two dyes may be demonstrated in the same cells, either side by side in granular form, or, under certain circumstances fused intimately to form purple granules within the phagocytes (2). Here there can be no question that the same group of cells is responsible for removing much of the dye from the plasma—a questionable assumption when widely differing substances are used in the study of "block-

ade." Highly colloidal dyestuffs such as brilliant vital red and Niagara sky blue have much to recommend them in studies of the sort here reported. The relatively slow elimination from the blood stream makes it easy to follow the process by quantitative methods. No doubt the desirability of such studies with dyes so similar in their general properties has occurred to many, but without the spectrophotometric method of analysis (3), it is well nigh impossible to make analysis of the purple plasma which results from the admixture of the two dyes.

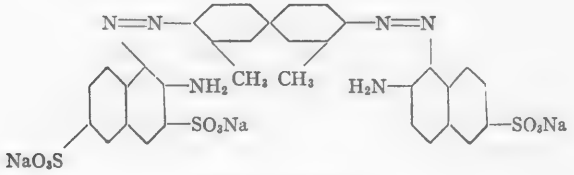
The experiments to follow give no evidence to show that the presence of one dye within the tissues influences in any manner the ability of the cells to take up and store another, and this is true regardless of whether the two dyes are given simultaneously or in sequence. Some of the dogs were vitally stained with repeated daily injections of brilliant vital red, after which a measured quantity of Niagara sky blue was injected, and the concentration of this dye in the blood stream determined over a period of several days, and it was found that it left the blood stream at almost identically the normal control rate. In other experiments the two dyes were injected simultaneously, and here again it will be shown that within limits of experimental error the tissues take up the blue dye at the normal rate, and this despite the fact that they were engaged at the same time in taking up the red dye also. Furthermore in the course of 72–96 hours the blue dye is leaving the blood stream very slowly—evidence of a state of equilibrium. The amount of blue dye now in circulation is almost identical with that observed in control experiments where no red dye was given. This shows that the final partition ratio between plasma and tissue is not influenced by the fact that red dye is present also. Presumably in these experiments the red dye is also distributed between plasma and tissues in a manner normally characteristic for that dye. Unfortunately the absorption curves of the two dyes are such that quantitative spectroscopic measurement of the red dye in the purple plasma is not so accurate as in the case of the blue one, and we found it impractical to follow the elimination of the red dye, though attempts along this line have shown that there is certainly no gross deviation from normal in the elimination of the latter.

Methods

Normal adult dogs maintained on a mixed diet were used. As in the previous experiments, feeding hours were so arranged that the plasma would show no lipemia during the hours when samples were taken for dye analysis. The two dyes used, brilliant vital red and Niagara sky blue, were obtained from the National Aniline and Chemical Company. The trade names for these dyes are those given them by that company. It is said that brilliant vital red corresponds chemically to brilliant congo R, rouge congo brillante R, brillantdianilrot R and azidin-scharlach R of other manufacturers. It is Schultz no. 370 and chemically is 1 mol of tolidine combined with 1 mol of β napthylamine, 3, 6, disulphonic acid and 1 mol of β naphthylamine, 6 monosulphonic acid. Niagara sky blue corresponds to Schultz no. 426 and chemically is 1 mol of dianisidine combined with 2 mols of H acid. It thus corresponds to dyes marketed by various firms under the names of diaminreinblau, bleu pur diamine, dianiblau H 6 G, oxaminreinblau 5 B, direktblau R B A, etc. The structural formulae are thought to be:



Niagara Sky Blue



Brilliant Vital Red

For purposes of injection these dyes were made up in a two per cent aqueous solution.

The blood samples were collected in well-vaselined "record" syringes and every precaution was taken to avoid hemolysis. The samples of blood were run into 15 cc. graduated hematocrit tubes containing 2 cc. of a 1.6 per cent solution of sodium oxalate to prevent clotting. After centrifugalization the amount of oxalated plasma in the tube was read off, thus enabling one to correct for the amount of oxalate solution present. The amount of dye in the plasma was determined by means of the spectrophotometer by a method (3) which allows quantitative analysis of each dye even when the two dyes are present together in the plasma.

EXPERIMENTAL

The data here reported were obtained from similar experiments carried out on 4 different dogs. The individual protocols are presented separately (experiments 45–48 incl.). Table 31 shows the results obtained in the individual cases. One of the experiments (experiment 46) was incomplete, and for this reason it was not used in making up the averages which are shown graphically in Charts 3A and 3B. For ease in presentation we shall refer chiefly to these curves where the progress of dye elimination can be seen at a glance. A careful study of the individual experiments shows minor variations, but all are alike in all essential respects.

Experiment 45. Dog 25–29. Male, Collie, 22 kg.

January 18, 1927. Twenty-four cubic centimeters of a 2 per cent solution of Niagara sky blue injected intravenously. At various intervals samples of blood were removed for analysis.

February 13, 1927. An intravenous injection consisting of 24 cc. of 2 per cent Niagara sky blue and 24 cc. of brilliant vital red was followed over a period of 4 days by frequent analysis of the plasma for Niagara sky blue. A sample of plasma taken immediately before making the double dye injection showed that the plasma had become free of the dye injected 3 weeks previously.

May 1 to 9th inclusive, daily injections of 24 cc. of a 2 per cent solution of brilliant vital red.

May 11. Twenty-four cubic centimeters of a 2 per cent solution of Niagara sky blue injected while the plasma still contained large quantities of brilliant vital red (about 400 mg. dye per liter plasma). Samples of blood taken during the next 4 days were analyzed for their content of Niagara sky blue.

The results of all of these analyses are shown in Table 31.

Experiment 46. Dog 25–35, Male, Setter, 18 kg.

March 30, 1927. Twenty cubic centimeters of a 2 per cent solution of Niagara sky blue injected intravenously. During the next 4 days samples of plasma were collected for dye analysis.

May 4, 1927. Plasma found to be free of dye. Intravenous injection of 20 cc. of a 2 per cent solution of brilliant vital red together with 20. cc. of a 2 per cent solution of Niagara sky blue. Samples were taken for analysis at intervals during the next 4 days.

The results of the analyses are shown in Table 31.

Experiment 47. Dog 25–16. Airedale, 19 kg.

January 19, 1927. Intravenous injection of 20 cc. of a 2 per cent solution of Niagara sky blue. The concentration of dye in the plasma was determined at intervals during the next 4 days.

February 11, 1927. The plasma was free of dye. Intravenous injection consisting of 20 cc. of 2 per cent brilliant vital red along with 20 cc. of 2 per cent Niagara sky blue. Analyses of Niagara sky blue in the plasma at intervals during the next 4 days.

May 3 to 11th. Daily intravenous injections of 20 cc. of a 2 per cent solution of brilliant vital red.

TABLE 31

	Mg. Niagara sky blue per liter plasma after					
	5 min.	1 hour	6 hours	24 hours	48 hours	96 hours
Experiment 45						
Niagara sky blue alone	360	240	170	46	32	22
Niagara sky blue after chronic staining with brilliant vital red	310	210	130	43	30	16
Niagara sky blue in presence of brilliant vital red simultaneously injected	290	210	115	50	43	30
Experiment 46						
Niagara sky blue alone	340	190	81	36	17	10
Niagara sky blue in presence of brilliant vital red simultaneously injected	335	205	79	39	20	14
Experiment 47						
Niagara sky blue alone	330	195	90	44	18	12
Niagara sky blue after chronic staining with brilliant vital red	320	190	94	30	14	9
Niagara sky blue in presence of brilliant vital red simultaneously injected	300	192	99	45	23	15
Experiment 48						
Niagara sky blue alone	320	175	75	40	25	12
Niagara sky blue after chronic staining with brilliant vital red	275	180	91	59	23	10
Niagara sky blue in presence of brilliant vital red simultaneously injected	303	175	91	65	38	11

May 13. The dog is deeply stained and the plasma contains large amounts of the red dye (about 390 mg. dye per liter plasma). Twenty cubic centimeters of a 2 per cent solution of Niagara sky blue injected intravenously. Analyses were made at intervals during the next 4 days to determine the amount of the blue dye in the plasma.

The results of all the analyses are recorded in Table 31.

Experiment 48. Dog 24–74. Female, Shepherd, 28 kg.

January 20, 1927. Intravenous injection of 30 cc. of a 2 per cent solution of Niagara sky blue. The concentration of dye in the plasma was determined at intervals during the next 4 days.

February 15, 1927. The plasma was free of dye. Intravenous injection consisting of 30 cc. of a 2 per cent solution of brilliant vital red along with 30 cc.

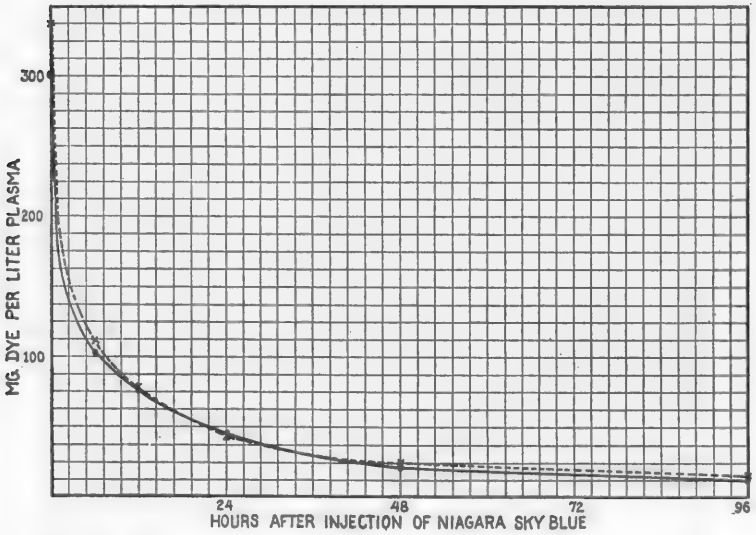


CHART 3A. Concentration of Niagara sky blue (average of 3 dogs). Interrupted line = unstained dogs. Continuous line = dogs previously stained with brilliant vital red.

of a 2 per cent solution of Niagara sky blue. Analyses of Niagara sky blue in the plasma at intervals during the next 4 days.

April 30 to May 8th inclusive, daily injections consisting of 30 cc. of 2 per cent solution of brilliant vital red.

May 10. The dog is deeply stained and the plasma contains large amounts of dye (about 320 mg. dye per liter plasma). Thirty cubic centimeters of a 2 per cent solution of Niagara sky blue injected intravenously. Analyses were made at intervals during the next 4 days to determine how rapidly the Niagara sky blue left the blood stream.

The results of all of the analyses are shown in Table 31.

Samples of centrifuged blood taken 5 minutes after an intravenous injection of brilliant vital red show plasma which is of a red color. If the dye present be measured colorimetrically it will be found that the amount present is directly proportional to the amount injected and inversely proportional to the total plasma volume of the animal. Similarly, an injection of Niagara sky blue imparts a bluish color to the plasma. Like brilliant vital red, this dye leaves the blood stream,

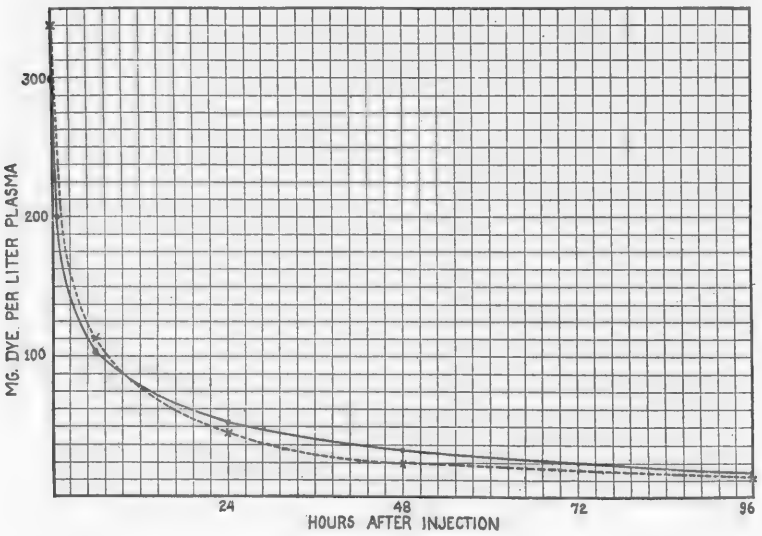


CHART 3B. Concentration of Niagara sky blue (average of 3 dogs). Interrupted line = Niagara sky blue injected alone. Continuous line = Niagara sky blue injected simultaneously with brilliant vital red.

and within 48 hours most of it is lodged in specialized phagocytic cells in various parts of the body. The rapidity with which each dye leaves the blood stream is somewhat different for each dye, and there are small individual differences in animals of the same species. With a given dye and under normal conditions the form of this curve is remarkably constant for a given animal.

We have given a series of four dogs an initial standardizing intravenous injection of Niagara sky blue. In each case the concentration

of dye in the plasma was measured five minutes later and again at intervals during the next 72 hours. A number of weeks were then allowed to elapse in order that the body might rid itself of the dye and be in a position to react normally once more. Each animal now received eight large daily injections of a two per cent solution of brilliant vital red. The tissues became progressively more red until staining was finally quite intense. To ascertain whether this red staining of the tissues reduces the ability of the latter to take up the blue dye from the plasma we then made an injection of the latter. We note from Chart 3A that, as in the original control experiment, the blue dye rises abruptly to a level of about 300 mg. per liter. From this point on until the end of the experiment 96 hours later the two curves are almost identical, showing quite unmistakably that these red-stained tissues will take up the blue dye just as effectively and as rapidly as do unstained tissues.

Some critic may wish to urge the point that some original phagocytic inhibition may have worn off before the test dose of blue dye was given. Indeed, it is held by many that dye-laden phagocytes may multiply by cell division or that new phagocytes may form from connective tissues with great rapidity and that these newly formed cells may take over the lost function of the stained cells, and thereby compensate for their deficiency. This point of view is thoroughly discussed by Aschoff (4). We must agree that some such increase in cells does occur when heavy injections are made repeatedly. We have also confirmed older observations that the individual phagocytes increase in size as they take up dye. But these data, presented in a paper to follow (2), include also some observations on double staining with these two dyes. It can be shown that when the two dyes are given in successive periods, the phagocytes contain granules of red and granules of blue dye, side by side. Very rarely indeed do we see cells which contain only one type of dye as we might expect if great numbers of new phagocytes were formed in the period of observation. The separate storage of materials in different cells reported by others need not signify that newly formed cells have been formed to take up the second substance; more likely is the view that the two substances used differed so widely that they simply found storage in cells which differed somewhat in type or in accessibility to the circulating blood.

We feel that purely morphological studies of these questions give only qualitative notions about the importance of cell multiplication in compensating for a hypothetical blockade. We are presenting certain physiological data which bear upon this point. The experiments were performed on the same dogs which we used in the experiments presented above. This second part of each experiment consisted in an effort to determine whether the tissues would take up Niagara sky blue at the normal rate if they were simultaneously busied with the phagocytosis of brilliant vital red. The animals were free of dye at the beginning of the experiment, but when the two dyes were mixed and injected we note that they gradually disappear from the plasma and become lodged largely within the tissues. It was thought that as the dyes accumulate together within the cells we might find a mutual antagonism—in other words, some evidence of failure of the cells to perform the double task of removing two dyes, each at its own normal speed. The fact that no such delay was observed is especially significant in these acute experiments. If phagocytic inhibition is to be observed at all we might expect surely to see it in these early stages of staining before cell multiplication could compensate for the filling of the cells with dye. If cell multiplication is responsible for the maintenance of phagocytic efficiency of the tissues we must be prepared to admit that the response is almost instantaneous—that there is little or no lag between incipient inhibition and compensatory multiplication and enlargement of cells.

In carrying out the experiments of which we have just spoken the two dyes were mixed in equal amounts and the mixture was injected into the blood stream. By means of the spectrophotometer we were able to measure the amount of each dye in the purple plasma which resulted. Samples of plasma were taken at intervals over a 72 hour period. From a technical point of view it is rather difficult to make precise measurements of brilliant vital red in this purple plasma, but the measurement of Niagara sky blue is much more easy and exact, for there is very little overlapping of the absorption bands at the red end of the spectrum where the blue dye must be measured. Furthermore the slight jaundice which sometimes develops does not interfere with the measurement of the blue dye as much as with the measurement of the red one. The concentration of the blue dye is shown in

Table 31 and graphically in Chart 3B. In this chart is also shown the normal control curve obtained some weeks previously by means of a simple injection of the blue dye alone. The two curves are almost identical throughout. The very slight retardation in elimination in the presence of the red dye is probably within the limits of experimental error. Like the experiments cited above, this experiment shows that a dye will pass from plasma into tissues at its own normal rate regardless of the presence of another dye. It is quite apparent that the tissues take up each dye in an independent manner.

DISCUSSION

We have already cited articles in which the reader will find discussions of the literature of "blockade" of the reticulo-endothelial system. It requires great generosity to admit much of the evidence which has been advanced in favor of the concept. We cannot but feel that much of the data is totally irrelevant. Changes in immune reactions, in fat metabolism, in pigment metabolism, and so forth, may well be associated with the injection of some foreign substance or with the use of some poison, and by some this is offered as evidence that the reticulo-endothelial system is blocked. Curiously enough this same evidence is offered by others to prove that the reticulo-endothelial system is concerned with immune reactions, fat or pigment metabolism. Obviously neither contention is built on firm foundation. Such rude procedures as these must cause great disturbances in the delicately regulated organism. Important changes in parenchymatous organs, changes in excretory activity of various organs are important considerations, and in our enthusiasm for the reticulo-endothelial system we must not neglect these other factors.

The rate at which dye leaves the blood stream depends upon excretion by liver and kidneys as well as upon the activity of the body phagocytes, nor, as we have shown, is it possible to disregard excretory activity by assuming it to be proportional to dosage. Such excretory activity must be measured carefully before we can assign to the phagocytes their proper importance in freeing the blood stream of dye, or of other materials. It is astonishing to see how generally this elementary precaution has been neglected. We have made some

progress in a study of the rate at which the liver excretes brilliant vital red, and certain of the data have been presented in the preceding paper. Unfortunately, Niagara sky blue is partially decolorized on reaching the bile, and for this reason it has not been possible to follow its elimination by the liver. The experiments presented above show that this dye leaves the blood stream at a normal rate, even though the body contains large amounts of red dye, and we have offered this as evidence that such red-stained tissues will take up the blue dye at a normal rate. Had the experiment turned out differently, and had we observed undue retention of the blue dye in the plasma, we would have been faced with the necessity of ascertaining whether defective phagocytosis or defective liver elimination was to blame, but since elimination is observed to be quite normal we do not need to postulate defective liver excretion. Indeed, if this could be shown to exist in this case, we would be forced to conclude that the red-stained tissues take up the blue dye *more* readily than normal, but there is no evidence to indicate that this is true, though this rather remote theoretical possibility must be kept in mind.

All things considered, we feel that brilliant vital red and Niagara sky blue are particularly well suited to studies on "blockade." They are not readily precipitated from solution by salts common in plasma or other body fluids and this is more than can be said of many substances which have been employed by others. The carbon of India ink is precipitated almost the moment it comes into contact with the plasma, and the rate at which these particles are filtered out of circulation gives us no clue about the activity of the phagocytic system. This same objection may be raised against the use of colloidal metals against the use of emulsions of fats and other substances.

The dyes we have used are free of these objections and we may confine our attention to storage by the tissues and to excretion from the body. These dyes are so much alike that they are taken up and stored within the same identical cells. With double staining experiments it is very rare to find cells which contain only one of the two dyes injected (2). It is quite otherwise when substances of widely different sort are used in combination. It is well known that the carbon particles of India ink do not pass readily through the capillary

walls. They soon become lodged within the phagocytes which are most directly related to the blood streams. We see them particularly in the Kupffer cells of the liver and in certain cells of the spleen. The enormous group of phagocytes in lymph nodes and in the connective tissues generally receive little or none of the carbon until a long period of time has elapsed. With such a substance there is no possibility of "blocking" more than a small part of the reticulo-endothelial system. One might well expect a normal response toward dyes and toward many other substances which readily reach cells which never come in contact with the ink. From such considerations we see the need of microscopic studies as controls in order that we may know at all times just which cells are concerned in any given process.

SUMMARY

When large amounts of brilliant vital red are injected into the blood stream of dogs, the dye is gradually removed from circulation, and most of it is deposited in numerous phagocytic cells which are scattered throughout various organs and tissues. The dye occurs largely in the form of tiny red granules crowded together in the cytoplasm of these cells.

If Niagara sky blue, a closely related dyestuff, is injected, it too is taken up and stored in these same cells. It is shown that the presence of red dye in the tissues does not inhibit the cells from taking up the blue one.

The normal ability of the phagocytes to take up Niagara sky blue is observed also when this dye is injected *simultaneously* with brilliant vital red. This normal response toward the blue dye is seen even though the phagocytes are busied at the same time in the process of engulfing and storing the red dye.

These experiments show that it is difficult if not impossible to "block" the cells with one dye so that their ability to take up another is even slightly impaired.

The two dyes employed in these studies are shown to be particularly suitable for experiments of the sort here reported.

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CUTANEOUS REACTIONS WITH CULTURE FILTRATES OF THE COLON TYPHOID TYPE

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HISTORICAL REVIEW

It has long been known that products of the typhoid bacillus induce a skin reaction. McKendrick (1) in 1923 carefully reviewed the early publications on the subject. In 1914 Gay (2) and his co-workers were able to demonstrate with the use of their so-called "typhoidin test" cutaneous manifestations, particularly in cases of recovery from typhoid fever irrespective of the antecedent date of the disease. The same reaction occurs only in relatively few instances in individuals who give no history of typhoid fever or typhoid vaccination. The original products of Gay were made in a manner similar to that used in the production of tuberculin and therefore contained many unknown substances which might greatly influence the results. Force and Stevens (3) improved the test by the use of an alcohol and ether dried typhoidin, precipitated from plain instead of from glycerol broth. By the use of this product Gay and Lamb (4) obtained improved results. However, considerable controversy has arisen as to the specificity of the test, since it was established by Nichols (5) that in individuals vaccinated against typhoid fever, 66 per cent reacted to paratyphoidin A and no more than 75 per cent to typhoidin. Nichols regarded a positive reaction as evidence of typhoid protein sensitization, which he thought was not synonymous with true disease immunity. Nichols and Hitchens (6) later concluded that several factors operate and should be considered in the interpretation of the results. In these they included allergy, toxicity, and non-specific factors such as personal idiosyncrasy. Similarly the work of Meyer and Christiansen (7) militated against the conclusion reached by Gay and his co-workers. Austrian and Bloomfield (8) also stated that normal persons and those who had had typhoid fever reacted in cutaneous tests with typhoidin; they, however, emphasized the value of the test when negative. Thompson (8) in 1921, applied three loopfuls of killed cultures to a clean and scarified area of the skin and noted that in a positive case there appeared to be continuance of the "red reaction" into the fourth day and commented on the possible value of the test for the detection of carriers.

McKendrick (l.c.) using standardized suspensions of typhoid bacilli with reference to agglutinability recorded that the procedure used by him constantly gave

positive reactions both in patients suffering from enteric fever and in chronic carriers. The test appeared to be highly specific. Early in convalescence the skin test became negative. Fleisher and Tierney (9) in 1926 used broth culture filtrates in 1:500 dilution and noted variability in the normal controls. In cases of the fever, the reaction was markedly diminished as compared with the weakly reacting normals or was negative. Early in the disease the reaction might be distinct, but later it became negative. Howell and Corrigan (10) also used broth culture filtrates and suggested that the reaction is allergic in nature, a positive reaction indicating immunity since those immunized or those with a history of typhoid fever, and those in the course of the disease tended to react positively in most instances; whereas only 40 per cent of other adults and 4 per cent of children gave a positive reaction.

In 1927 Ecker and Rimington (11) showed that synthetic medium culture filtrates of the aertrycke type of paratyphoid bacilli, concentrated in vacuo and dialyzed, when injected into the skin of normal (non-sensitized) rabbits produced a raised edematous and reddened area. After decomposition of a lead precipitate of the same filtrate material by means of $(NH_4)_2 SO_4$ and dialysis, a marked reaction was also obtained by a similar injection, the wheal being about 1.5 cm. in diameter within 24 hours. The center was about 4 mm. in diameter, elevated and white, and the surrounding areas was hyperemic. In about 4 days the central area became necrotic and the hyperemia subsided. The original filtrate (not inoculated, concentrated, or dialyzed) failed to give a reaction. The concentrated products gave certain color reactions which are usually associated with the presence of carbohydrates. Small quantities of nitrogen were also obtained (0.3 to 0.4 per cent). So far as we know this observation is the first one correlating bacterial carbohydrates with toxicity.

Up to 1928 the demonstration of skin reactivity with filtrates had been accomplished only by intracutaneous injections. Shwartzman (12), however, in that year demonstrated a new phenomenon. He observed that following an intracutaneous injection of the filtrate (tryptic digest broth) of the typhoid bacillus an intravenous injection caused a hemorrhagic area to appear at the point of the original intracutaneous injection. He has produced the phenomenon with filtrates of a large number of organisms. The reactions were fully developed after 4 or 5 hours following the intravenous injection of the filtrate. This author was able to neutralize the filtrate to be used for the intravenous injection with the specific immune antityphoid serum.

In his early work Shwartzman used a tryptic digest broth but to avoid extraneous materials he later used growths on veal infusion agar, washing the organisms from the surface with 0.9 per cent saline. The centrifuged suspensions were then passed through Berkefeld "N" candles, but this filtrate gave very little reaction intracutaneously. He further noted that the filtrate was inactivated only by autoclaving. Dilutions as high as 1:48 when injected into the skin prepared it for the "Shwartzman phenomenon." Two months after the publication of Shwartz-

man, Hanger, Jr., (13) reported that when animals showing a positive skin test to filtrates of B. lepisepticum received an intravenous injection of the filtrate, after several hours obliteration did not occur but an intensification of the local reaction was found, and the site of inoculation often showed blue discoloration with areas of petechial hemorrhages. In most instances, an extensive local necrosis occurred. The author stated that he has never observed the phenomenon except among rabbits naturally allergic or rendered allergic by a previous infection. The reaction was proportional to the allergy of the animal to the filtrate used and appeared to be non-specific. He further stated that it is rather obscure why the filtrate diluted in the blood is more destructive to endothelium than the same filtrate concentrated locally. The intensified reaction described by Hanger appears to be the same type of reaction first described by Shwartzman. In a previous paper (same year) Hanger (13) showed that rabbits are often naturally allergic to filtrates of many gram negative bacteria but in this report no mention was made of the intensified (hemorrhagic) reaction.

In our work we have attempted to procure as pure products as possible in order to avoid extraneous materials which might introduce confusion. The method described by Ecker and Rimington was used to produce the pure filtrates. We have studied the effect of heat and light on the production of a skin reaction with the concentrated filtrates; and the possibility of producing an immunity to these filtrates by various methods: (a) intravenously, (b) intravenously with massive doses of the concentrated filtrates and (c) intracutaneously. Attempts were made to study and to explain the mechanisms involved when the toxic filtrates are mixed with the homologous antisera and introduced into the skin. We watched carefully the cutaneous reactions obtained in the immunized animals. To determine whether or not specificity existed "crossing over" experiments were made in vivo. Experiments were also made to observe the effect of normal human and convalescent typhoid sera on the filtrates intracutaneously injected. Attempts were made to note the relation of the Shwartzman products to ours, and whether or not filtrates from enterococci would induce the Shwartzman phenomenon in animals prepared with filtrates of the colon-typhoid group of organisms. Incidentally all the sera of the immunized animals were examined for the presence of precipitins.

Method

Seven members of the colon-typhoid group of organisms, namely, 1 B. typhosus, 1 B. paratyphosus A, 2 B. paratyphosus (aertrycke type), 1 B. enteritidis, 1 B.

paratyphosus B. (Schottmüller), and 1 B. coli were employed. The organisms were plated out and the smooth colonies—known to be the best producers of toxic filtrates (Ecker)—isolated in all cases. They were then made to grow by repeated transplantations on the medium of Cahn-Bonner (See Ecker and Rimington, l. c.). After five days of growth the pure cultures were centrifuged and passed through Berkefeld "N" candles. The bacteria-free filtrates were then concentrated in vacuo and dialyzed in collodion sacs against running distilled water until salt free with $AgNO_3$.

Strict asepsis was observed. The concentrated and dialyzed filtrates were made isotonic with 0.9% NaCl and ampouled. The ampoules were then heated at 60°C. for one hour and tested for sterility.

As controls the same medium (to which *no* organisms were added) was filtered, concentrated, dialyzed, and made isotonic. As a further check the condensate and dialysate were treated in a like manner. Finally in a large series of our rabbits sterile 0.9% NaCl was injected intracutaneously. The controls were negative in all cases.

The toxicity of the filtrates was determined by intravenous injections of rabbits. The typical picture described by Ecker and others was demonstrated. All the rabbits used weighed from 2000 to 3000 gms. The 17 animals used for immunization purposes were given injections over a period of ten weeks as follows:

(1) Intravenous injections with increasing doses of the dilute and concentrated filtrates starting with 0.3 of one cc. (dilute) and ending with 1.5 cc. of the concentrated filtrates.

(2) Intravenous injections with massive doses of the concentrated filtrate (4 injections of 5 cc. each over a period of 20 days).

(3) Intracutaneous injections of about 11 cc. given in 0.2 cc., doses totaling approximately 55 injections over a period of 10 weeks.

All the rabbits were bled by cardiac puncture, 11 weeks after the beginning of the immunization. Prior to the puncture food was withheld for at least 24 hours so that clear sera for precipitin tests would be obtained.

Finally all the immunized animals were given intracutaneous injections of the homologous and heterologous filtrates.

Where attempts were made to neutralize the toxic filtrates by the homologous immune sera the mixtures were incubated for 2 hours prior to the intracutaneous injection.

All the animals used for the intracutaneous injections were depilated on both flanks with a mixture of barium sulphide, zinc oxide, and starch to avoid irritation. The animals in most cases were injected 24 hours after depilation.

EXPERIMENTAL

(*I*) *Effect of Heat and Light on the Filtrates.*—The filtrates were exposed to heat and ultraviolet light to observe whether or not their

toxicity was diminished. The size and character of the wheals were noted and tabulated. The results of the experiments are given in Table I.

From this table it is seen that in all cases the controls were positive, the wheals varying in size in different animals. It will be further

TABLE I

Showing the Effect of Heat and Ultraviolet Light on the Toxicity of the Filtrates

Filtrates of	Control reaction	Heated at 100°C. for 20 minutes	Autoclaved at 15 lbs. for 15 min.	Ultraviolet light "C" carbon 15 minutes (50 cm.)	Ultraviolet light "C" carbon for 30 minutes
B. typhosus	Wheal 12 x 12 mm. red +	Wheal 11 x 10 mm. red +	−	Wheal 12 x 15 mm. red +	±
B. paratyphosus A	Wheal 14 x 12 mm. red +	Wheal 11 x 9 mm. red +	−	Wheal 10 x 8 mm. red +	−
B. coli	Wheal 17 x 12 mm. red +	Wheal 11 x 9 mm. red +	−	Wheal 10 x 8 mm. red +	−
Aertrycke 185 B. paratyphosus	Wheal 8 x 6 mm. red +	Wheal 7 x 5 mm. red +	−	Wheal 6 x 7 mm. red +	−
(Deli B. enteritidis)	Wheal 15 x 14 mm. red +	Wheal 11 x 18 mm. red +	−	Wheal 13 x 12 mm. red +	±
Aertrycke de Nobele B. paratyphosus	Wheal 12 x 11 mm. red edematous +	Wheal 8 x 6 mm. red +	−	Wheal 6 x 6 mm. red +	±
B. paratyphosus B. Schottmüller type	Wheal 10 x 8 mm. elevated red +	Wheal 6 x 5 mm. red elevated +	−	Wheal 9 x 8 mm. red +	−

noted that heating at 100°C. for 20 minutes did not destroy completely the toxicity of the filtrates. Autoclaving, however, at 15 lbs. for 15 minutes, destroyed or inhibited the reaction-producing material in all seven filtrates. Ultraviolet light with a "C" carbon (see Perkins and Welch (14)) with an exposure in quartz tubes of 30 minutes at 50 cm. distance using 55 volts and 30 amperes completely

inhibited or destroyed the toxicity in 4 cases. In three cases a questionable reaction developed. Exposure of 15 minutes under the same conditions failed to weaken the potency of the filtrate.

(2) *Effect of Dilution on the Toxicity of the Filtrates.*—The seven concentrated filtrates were diluted, as shown in Table II, with 0.9% NaCl, and 0.2 of one cc. was inoculated intracutaneously. It will be

TABLE II

Showing Endpoint by Dilution of Concentrated and Dialyzed Filtrates

Filtrates of	Dilutions					
	0	1:2	1:4	1:8	1:10	1:16
B. typhosus	+	+	+	−	−	−
B. paratyphosus A	++	++	+	±	−	−
B. coli	++	++	+	±	−	−
Aertrycke 185 B. paratyphosus	++	++	++	+	+	±
B. enteritidis (Deli)	++	++	++	+	+	±
Aertrycke de Nobele B. paratyphosus	++	++	+	±	−	−
B. paratyphosus B. Schottmüller type	++	++	++	+	+	+

++ large, red and edematous.
+ definite red area.
± Small pale, pink area.
− No reaction.

seen that the loss of ability to produce a skin reaction varied for the different filtrates; the B. paratyphosus Schottmüller, aertrycke 185, and B. enteritidis strains remained positive when diluted 1:16; the B. paratyphosus A, B. coli, and aertrycke de Nobele were positive at 1:8; the B. typhosus strain was positive only in a dilution of 1:4. See Table II.

(3) *Effect of $CaCl_2$ in 1:500 Dilution.* (*Kataphylaxis*).—Introduc-

tion of $CaCl_2$ in 1:500 dilution in saline which in itself produces no visible reaction in the skin, was found to increase the activity of the filtrates when injected into the same area. The reactions persisted from four to six days but the control reactions with the filtrates usually disappeared in about three days.

(*4*) *Effect of Freezing on Filtrates.*—Freezing of the filtrates until solid with subsequent gradual thawing indicated a possible method for concentration, inasmuch as the part which melted first caused typical reactions. This method, because of its practical significance, is being studied further in this laboratory.

Immunization

(*1*) *Size and Character of Wheal before, during, and after Immunization.*—During immunization, by the intracutaneous method, the size and type of the wheals were carefully determined. It was observed that there was a definite decrease in size and character (less red) of the wheals. Originally they measured from two to three cm. in diameter, showing a deep red, edematous area, but as time went on the areas decreased in size until following the final injection (10th) the size of the wheals had diminished to from 5 to 7 mm. in diameter. However, it was also noted that whereas the wheal at first appeared flat, edematous and red, it now became nodular, definitely showing an *indurative* character. In one animal a row of seven nodules (7 injections of 0.2 of one cc. each, the 6th intracutaneous injection) persisted for over one week. Animals that received massive doses of the concentrated filtrates by vein also showed a similar indurative reaction, when they received, after immunization, the homologous filtrates intracutaneously.

In spite of the fact that 2 to 3 cc. of the original filtrate (dilute) caused death of a large number of normal animals, it was found that immunized animals could tolerate massive doses (5 cc.) of the concentrated and dialyzed filtrate. This is interesting since 5 cc. of the concentrated filtrate represent 50 cc. of the original filtrate.

(*2*) *Precipitin Content of Sera.*—All the sera have been tested for their precipitin content by the ring method in Hektoen tubes. As antigen we used both the concentrated and the original dilute filtrate. On testing the sera from animals that received the filtrate intracutaneously no precipitins were demonstrated.

The sera of animals that received the filtrates by vein in increasing and finally in massive doses all showed precipitating qualities in varying degrees. The strongest reactions were found in the sera of animals that received the filtrate from the typhoid bacillus and from the B. paratyphosus aertrycke, type "de Nobele." The other five sera gave moderate but definite rings with the dilute filtrates.

The sera obtained from animals that received massive doses of filtrates from the typhoid bacillus, aertrycke 185, and B. paratyphosus A. gave the most marked precipitin reactions. In all cases a three plus reaction was obtained when using both the dilute and the concentrated filtrates as antigen. All the tubes were given at least 20 hours at room temperature before a final reading was taken, and in no instance did salt solution cause a ring to be formed.

(*3*) *Neutralization of Toxic Filtrates by Normal Human, Convalescent Typhoid and Rabbit Sera.*—We secured three sera from human typhoid cases. Of these one (A) was from a convalescent 5 weeks after the onset of the disease; the second (B), and the third (C) were from cases that gave a negative Widal but from which the organisms had been obtained. The serum from (B) was obtained 4 weeks after the onset of the disease and from (C) during the first week.

Two methods of neutralization were attempted: First, increasing amounts of the sera mixed with our dilute and concentrated filtrates (typhoid) were injected intracutaneously, and second, the sera alone were injected into the skin and 24 hours later, the filtrates into the same areas. Each serum was tested on one animal. In all cases we were unable to demonstrate a neutralization of the toxic effect. The wheals were larger than the controls, deeper red and hard. There was a definitely, indurated area. We injected in these instances the antigen (antibody complexes) into the skin.

Using the same method but now with six normal human sera (two obtained from cases of hypertension) we found that in all cases, except one, the reactions were of the same size and color as the controls. In the one normal serum which gave no reactions with the filtrate we demonstrated agglutinins in a dilution of 1:64, whereas the others were all less than 1:15.

As a further control a rabbit antityphoid serum from which a serum titer 1/640 was obtained and 4 other normal rabbit sera were

tested by the two methods just mentioned. The immune serum definitely increased the size of the wheals when injected with its homologous filtrates and the areas were red, (raised and indurated). When the normal rabbit sera were individually mixed with each of the seven filtrates, we observed definite areas but of a different character than that of the immune rabbit serum causing wheals which were red, flat and edematous, with no induration. It is therefore to be assumed that during the course of the immunization with a whole typhoid culture of the animal, we have also sensitized the animal to the bacterial products. Further studies along this line will be published at a later date.

(*4*) *Effect of the Anti-Filtrate Sera on the Filtrates.*—Seventeen anti-filtrate sera were employed. Of these seven were prepared by intravenous injections of increasing doses, three by intravenous injections of massive doses, and seven by intracutaneous injections of the concentrated filtrates. All the sera were inactivated at 55°C. for 20 minutes. The sera were mixed with the homologous filtrates in equal portions and incubated for two hours at 37°C. The mixtures of sera and homologous filtrates were injected intracutaneously into a series of rabbits. The results were as follows:

(A) The sera from the animals that received increasing doses of the filtrate intravenously mixed with the concentrated filtrates caused raised indurated and reddened areas.

(B) The three sera obtained from animals that received massive doses mixed intravenously with the concentrated filtrates and injected caused the appearance of red, edematous and indurated wheals.

(C) The sera from the intracutaneously treated rabbits mixed with the filtrates caused slightly edematous, red areas but no indurations were noted. (These sera had no precipitins.)

Two of these animals (from A) were then given 5 cc. of the concentrated filtrates (B. coli and B. paratyphosus aertrycke) by vein to produce a Shwartzman reaction. In four to five hours after the injection of the filtrate a marked hemorrhagic, blue-black area appeared at the point of former injections.

(*5*) *Specificity by the "Shwartzman Phenomenon" in the Group.*—Each of seven rabbits received the seven filtrates intracutaneously in the usual 0.2 of one cc. doses. The initial reactions were about 10 to

TABLE III

Showing the Effect of the Intravenous Injection of Seven Different Filtrates in Rabbits Previously Injected with all the Filtrates Intracutaneously (Shwartzman Technic)

Rabbit No.	Filtrate injected intravenously 24 hrs. after intracutaneous injection	Dose in cc.	Intracutaneous injections with 0.2 cc. of filtrates of the following filtrates						
			B. typhosus	B. paraty. A.	B. coli	B. paraty. 185. Aertrycke type	B. enteritidis	Aer. de Nobele B. paraty. B.	Schottmüller B. paraty. B.
3	B. typhosus	5	—	12 x 12 mm. dark red	4 x 7 mm. red	—	4 x 7 mm. blue hemorrhagic	—	—
8	B. paraty. A.	4	Original area dk. red	Original area dk. red	Original area dk. red	Original area dk. red	Original area dk. red	Original area —blue in color	—
7	B. coli	4.5	5 x 5 mm. blue	5 x 5 mm. blue	25 x 15 mm. blue with dk. red center	—	Original area darker red	5 x 5 mm. blue	—
2	B. paraty. B. aer. 185	5	4 x 5 mm. red	—	4 x 5 mm. red	—	4 x 5 mm. red	—	—
5	B. enteritidis (Deli.)	5	8 x 12 mm. purple (hemorrhagic)	—	12 x 12 mm. Purple, Intracutaneous hemorrhages	Original area pale blue	12 x 14 mm. purple severe cutaneous hemorrhages	—	4 x 4 mm. Purple and markedly hemorrhagic

1	B. paraty. B. aer. de Nobele	5	—	8 x 8 mm. blue moderately hemorrhagic	5 x 5 mm. Pale blue mildly hemorrhagic	5 x 5 mm. Pale Blue	—	—	—
6	B. paraty. B. Schottmüller	3	—	—	—	—	—	—	—

12 mm. in diameter. They were edematous, flat and red. Twenty-four hours later each animal received one of the seven filtrates (3 to 5 cc. concentrated) by vein. The results are tabulated in Table III.

From this table it is apparent that the filtrate from B. paratyphosus Schottmüller type has failed to produce a reaction. This is probably due to the fact that only 3 cc. were given by vein or possibly to variability of the animal. When five cc. were used, the reaction appeared in all cases. The other filtrates have caused more or less severe reactions, the homologous areas being often markedly hemorrhagic. The experiment shows that although the homologous filtrates usually causes a typical Shwartzman reaction to appear, heterologous filtrates of the same group will also in some cases produce the phenomenon. We were primarily interested in comparing our filtrates with those of Dr. Shwartzman, who kindly sent us some of his B. typhosus filtrates. To test the relationship we injected into the skin of two animals, 0.2 of one cc. of Shwartzman's typhoid filtrate and 0.2 of one cc. of our own typhoid filtrate. After a period of 24 hours, our filtrate showed a definite red wheal; whereas Shwartzman's filtrate showed only a pale pink area. One animal was now given 5 cc. of the Shwartzman product and the other, 5 cc. of our filtrate. All four of the intracutaneous reactions had changed character within 5 hours. The area now appeared hemorrhagic, measuring 10 x 10 mm. in diameter. It seems therefore since his product has the capacity to prepare the skin for our product and, vice versa, our filtrate prepares the skin for his product, that we are without a doubt dealing with the same factor or factors. The only difference between the two is that with our own filtrates we always obtain a red, edematous reaction with the intracutaneous injection. This we now know is due to concentration of the filtrates.

As controls we gave the second injection subcutaneously but no hemorrhagic areas developed.

Similarly broth culture filtrates from three serologically different types of enterococci of Welch (15) were injected intravenously into a series of three animals whose skin had been prepared by all our seven filtrates. No hemorrhagic areas developed, definitely demonstrating at least a group specificity. Broth alone or saline containing 1 per cent nucleic acid were also injected into prepared animals with negative results.

DISCUSSION

Considerable controversy has arisen as to the value of skin tests in the course of typhoid fever. The methods of preparation of the reacting agents have been many and varied, and the mechanism involved little understood. In most instances filtrates of complex media cultures were employed, rather than suspensions of the organisms or fractions thereof. It must be assumed that during the course of the disease and probably also in the carrier state bacterial allergy exists, and little is known concerning the development and persistence of allergic state in this disease. That the reactions may be allergic is not a new conception.

In the present study we paid careful attention to the production of a bacterial colloid, free from all possible extraneous materials. We showed that with the concentrated synthetic medium culture filtrates of Ecker and Rimington, erythema and edema can be readily produced in the rabbit's skin; while the original, unconcentrated filtrate, rarely produces an inflammation. Immunization experiments by the intracutaneous and the intravenous route definitely demonstrated a reduction in the reaction but obliteration was not noted and at the same time an indurative reaction was observed. The mechanism therefore is one of antigen-antibody reaction in the tissues, yet this has not produced a hemorrhagic area as in the case of the "Shwartzman phenomenon" when the antigen is introduced by two different routes, (skin and vein). Indurations were also seen when the sera of convalescent typhoid patients were introduced into the skin together with the filtrate. Whether or not the original reaction with the concentrated filtrate is also due to an allergic state is difficult to say but it is a known fact that gram negative bacilli of this group are constant inhabitants of the gastrointestinal canal and this may explain the appearance of the reaction.

We have succeeded in producing the "Shwartzman phenomenon" with our concentrated filtrates, thus confirming these observations with a highly purified product. The reactions were further obtained in animals whose skin was prepared by Shwartzman's filtrate and by our own filtrate and vice versa, indicating that we must be dealing with the same reacting products. The Shwartzman reaction also

occurred in animals in which the skin preparation was made by the intracutaneous injection of filtrate and antiserum, that is, at the site of an inflammatory process on the skin, a hemorrhagic area would appear if followed by an intravenous injection of the filtrate alone.

A single injection of the filtrate and its homologous antiserum never produced a hemorrhagic area. The reason that an intravenous injection of the filtrate is necessary for the production of the reaction is difficult to explain. Subcutaneous injection of the filtrate failed to produce the phenomenon. Filtrates of cultures of organisms of the same group will produce the reaction; whereas filtrates from non-related organisms (enterococci) failed to react in animals whose skins were prepared by filtrates of all the members of the colon-typhoid group.

The products used by us were relatively thermostabile but did not withstand autoclaving for 15 minutes at 15 lbs. pressure, neither did they withstand 30 minutes exposure to ultraviolet light.

Ecker and Rimington have reported on the chemical nature of these products and similar findings were also obtained by Branham (16) and others.

SUMMARY AND CONCLUSIONS

The observations of Ecker and Rimington on the production of skin reactions with synthetic medium culture filtrates (concentrated and dialyzed) of organisms belonging to the colon-typhoid group have been confirmed and extended.

The filtrates show a marked degree of thermostability and marked resistance to ultraviolet radiation.

Intravenous injections of the filtrates stimulate the production of precipitins. No precipitins are produced in the sera of rabbits that receive intracutaneous injections only.

At no time during the course of immunization was the skin reaction obliterated. An indurative process was noted at the site of injection (skin) during the course of immunization. Mixtures of the filtrates with their homologous antisera, obtained from animals immunized by the intravenous route, and injected into skin, also failed to obliterate the erythema and edema.

The observations of Shwartzman have been confirmed; the Shwartz-

man phenomenon appears to be group or species specific. Filtrates from enterococci failed to produce the phenomenon. Filtrates ot other unrelated organisms have not been studied.

The products of Shwartzman and of Ecker and Rimington appear to be the same.

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CHANGES IN HUMORAL IMMUNITY OCCURRING DURING THE EARLY STAGES OF EXPERIMENTAL PNEUMOCOCCUS INFECTION*

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While much has been learned concerning the appearance of immune substances in the blood at the time of recovery from lobar pneumonia and experimental pneumococcus infection, relatively little is known about changes in humoral immunity occurring during the early phases of the disease. That such changes occur and have an important bearing on the inception and evolution of the infection seems highly probable in the light of recent experimental evidence as to the nature of natural resistance to pneumococcus infection (1).

The findings of earlier workers in this field are inconclusive. This is to be explained presumably by the use of methods lacking sufficient delicacy for the detection of normal anti-pneumococcus substances in the blood stream. Wolf (2), Rosenow (3), Tunnicliff (4), and others, testing phagocytic activity by the determination of the opsonic index (Wright), agreed for the most part that the opsonic index was low in severe pneumonia cases at the height of the disease and was followed by a slight rise at the time of crisis. In fatal cases there was a fall in the opsonic index before death. However, these workers used avirulent strains of pneumococci in their tests; whereas the organisms isolated from pneumonia patients were almost invariably virulent and found to be resistant to the opsonic action of the serum. Other workers (5), (6), (7), studying the humoral changes in lobar pneumonia, were unable to demonstrate opsonins, agglutinins, or mouse protective substances, in the blood stream until about the time of crisis. Bull (8), on the other hand, studying the natural resistance of the dog to pneumococcus infection, was able to demonstrate clearing power of normal dogs blood for highly virulent pneumococci. He injected dogs with virulent pneumococcus cultures intravenously and by plating samples of blood at short intervals observed a rapid decrease in the number of pneumococci in the blood stream following injection.

* This work has been conducted under a grant from the Douglas Smith Foundation for Medical Research of the University of Chicago.

In fatal cases this diminution was only temporary and was followed by an increase and death from septicemia. He inferred that the clearing of the blood was due to agglutination and the phagocytic action of the circulating leucocytes and the cells of the reticulo-endothelial system, although neither opsonins nor agglutinins could be demonstrated in the dog's serum until the sixth or seventh day of the disease.

Tillett (9), working with Type III pneumococcus, using a similar technique, injected rabbits intravenously and showed that avirulent or R forms were rapidly taken out of the blood stream. With strains of slight virulence, there was a decrease and a subsequent rise in the number of circulating pneumococci. When highly virulent strains were used, there was no diminution but a steady increase in the bacteremia until death of the animal. With the usual methods employed for the demonstration of opsonins and agglutinins, Tillett failed to find evidence of humoral immune properties sufficient to account for the rabbit's ability to destroy the avirulent pneumococci.

Recently Robertson and Sia (10) (11), studying the natural resistance of animals to pneumococcus infection, found by an especially devised technique that the serum and leucocytes of pneumococcus resistant animals such as the dog, cat, pig, etc., possessed marked pneumococcidal properties for highly virulent strains of pneumococci while the blood of pneumococcus susceptible animals as the rabbit and guinea pig was lacking in this action. This property of the serum and leucocytes of resistant animals was found to depend on the presence of opsonins and agglutinins which could be demonstrated by employing a relatively large ratio of serum to microorganisms (1).

The technique of Robertson and Sia, by which it is possible to demonstrate constantly natural humoral immune bodies in the blood of certain animal species, provides a means of studying changes in the circulating defense mechanism during the inception and evolution of pneumococcus infection. The object of the present study has been primarily to follow the early changes in the humoral immune substances of the dog and cat during experimental pneumococcus infection.

Methods

Infection: Normal dogs and cats were infected by intrapleural or intraperitoneal injections in the first experiments and by intrabronchial insufflation in the later ones. A virulent Type I strain and a virulent Type II strain of pneumococcus originally isolated from pneumonia cases were used. The virulence was maintained by frequent passage through rabbits.

Serum: A blood specimen was drawn before the animal was infected and daily specimens were taken until death or recovery. The blood was allowed to cool in

the ice box and the serum was withdrawn later. Blood cultures and plate counts were done on most of the animals. All sera that contained pneumococci were filtered through an especially designed filter.[1] The sera were preserved in the ice box in bottles containing carbon dioxide.[2] All sera from one animal were tested at one time not later than seven days after the first blood specimen was drawn.

Opsonic Test: The method described by Robertson and Sia (1) was followed closely. Homologous leucocytes were obtained from aleuronat exudates withdrawn from the pleural cavity 15 to 18 hours after injection of the aleuronat. Type I pneumococcus was used in the lag phase of growth and Type II pneumococcus in the active growth phase. The pneumococci were sensitized for periods of ½ hour for actively growing cultures and one hour for cultures in the lag phase in the serum to be tested. A ratio of 50 parts of serum to one part of pneumococcus suspension was employed. The organisms were then sedimented by centrifugation at high speed for ¾ hour; the serum was removed and the pneumococci were taken up in sufficient Locke's solution to make a suspension somewhat more concentrated than that originally added. Wright's capillary pipette mixtures were made using equal parts of sensitized pneumococcus suspension, 1 to 5 normal serum, and a standard leucocyte suspension. The pipettes were incubated 45 minutes and smears were made which were stained with Cross' stain. 100 leucocytes were counted and the percent showing phagocytosis noted.

Pneumococcidal Test: The pneumococcidal promoting substances of the serum were tested by the method described by Robertson and Sia (10). 0.1 cc. dilutions of an actively growing pneumococcus suspension were added to small tubes containing 0.3 cc. serum and 0.1 cc. of a standard leucocyte suspension. The tubes were sealed with corks dipped in paraffin and rotated on an agitator for 15 to 18 hours. Readings were made by determining the amount of methemoglobin formed at the end of 18, 42, and 72 hours. Smears were then made to determine the survival of pneumococci.

EXPERIMENTAL

Generalized Pneumococcus Infection

Experiment I: Cat No. 1, weighing 4000 grams, was bled 10 cc. from the heart and four hours later 0.5 cc. Type II pneumococcus culture was injected intrapleurally. 18 hours subsequently the cat's temperature was 41°C. and it appeared ill. Death occurred on the fourth day. Autopsy revealed peritonitis and a massive empyema of both pleural cavities. The lungs were not consolidated. Culture of the heart's blood was positive for penumococci. The collected sera were filtered to eliminate any pneumococci present and the opsonic activity of the serum was determined.

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[1] Small pressure filters were made, using the Seitz principle.

[2] According to McAlpine and Valley (12) alexin or complement can be preserved for a long period of time in an atmosphere of CO_2.

The results are shown in Figure I. The serum of the cat drawn before infection showed marked opsonic activity; 67 per cent of the leucocytes counted showed phagocytosis. 18 hours after infection

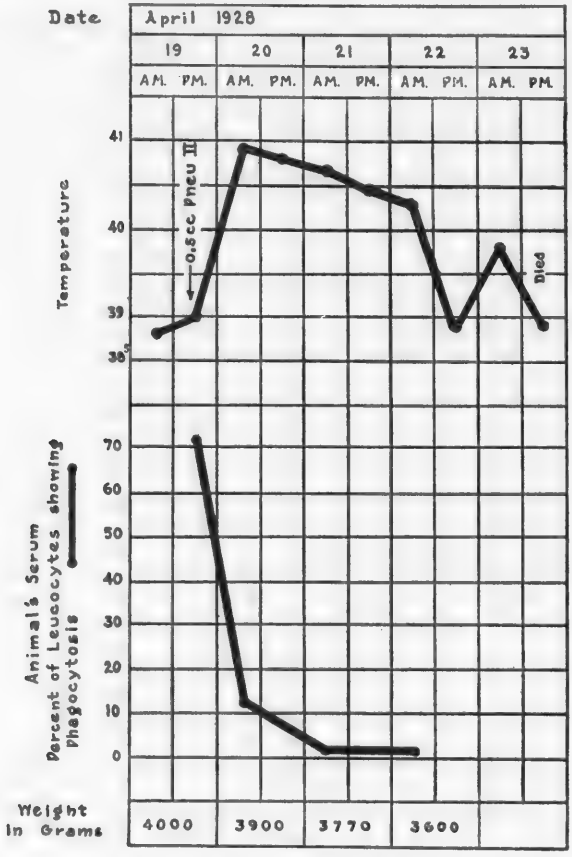

FIGURE I. Cat No. 1. Experimental pneumococcus infection following the intrapleural injection of 0.5 cc. Type II pneumococcus culture.

the opsonic titer had diminished markedly; only 12 per cent of the cells showed phagocytosis. After 48 hours this serum property had disappeared.

Experiment II: Dog No. 1, weighing 20.9 kg., was bled 15 cc. from the heart and 4 cc. of an actively growing Type I pneumococcus culture were injected into the right pleural cavity. The next morning the dog appeared sick and the temperature had increased to 41°C. Blood cultures at 18 hours and 42 hours after

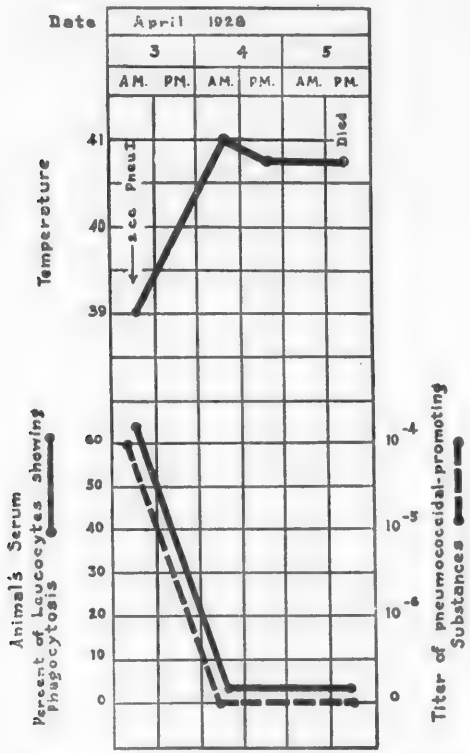

FIGURE II. Dog No. 1. Experimental pneumococcus infection following the intrapleural injection of 2 cc. Type I pneumococcus culture.

infection were positive for pneumococci. The dog died on the third day. Autopsy revealed a massive empyema of both pleural cavities. The lungs were not consolidated.

Before infection the serum contained a high concentration of opsonins. Phagocytosis was noted in 65 per cent of the leucocytes

counted with 60 per cent showing five pairs or more of pneumococci. 18 hours after the inception of infection the opsonic content of the serum had dropped to a point where only 5 per cent of the leucocytes showed phagocytosis with no cells containing more than one or two pairs of pneumococci (Figure II). The pneumococcidal promoting action of the serum showed a similar fall in activity (Table I). The serum (0.3 cc.) taken before infection was capable of causing destruc-

TABLE I

Effect of Pneumococcus Infection on the Pneumococcidal-Promoting Activity of the Serum

Dog serum 0.3 cc. + normal dog leucocytes 0.1 cc. + pneumococcus suspension 0.1 cc.

Kind of serum	Amount of standard suspension		Growth as shown by color changes			Survival of pneumococci stained film
			17 hrs.	42 hrs.	72 hrs.	
		cc.				
Serum before infection	10^{-2}	(0.01)	+++	++++	++++	+
	10^{-3}	(0.001)	++	+++	++++	+
	10^{-4}	(0.0001)	0	0	0	0
	10^{-5}	(0.00001)	0	0	0	0
	10^{-6}	(0.000001)	0	0	0	0
Serum 18 hrs. after	10^{-4}	(0.0001)	++++			+
	10^{-5}	(0.00001)	++++			+
	10^{-6}	(0.000001)	++++			+
	10^{-7}	(0.0000001)	++++			+
Serum 42 hrs. after	10^{-5}	(0.00001)	++++			+
	10^{-6}	(0.000001)	++++			+
	10^{-7}	(0.0000001)	++++			+

tion in the serum-leucocyte mixture of approximately 100,000 pneumococci (10–4 of the standard suspension) but 18 hours after infection it had lost this property.

Experiment III: Dog No. 2, weighing 8.2 kg., was bled 15 cc. from the jugular vein and 2 cc. of an actively growing culture of Type I pneumococcus were injected into the trachea by a needle and syringe. The dog was held so that the culture would run into the right lung. 18 hours later the dog's temperature had risen to 41.1°C. and it appeared very toxic. Death occurred on the third day. Autopsy

revealed an empyema of both pleural cavities. The lobes of the right lung were well collapsed and markedly congested. The left lung resembled the right except that it was more air containing. Microscopical sections showed congestion of the alveolar walls but no leucocytic exudate was present.

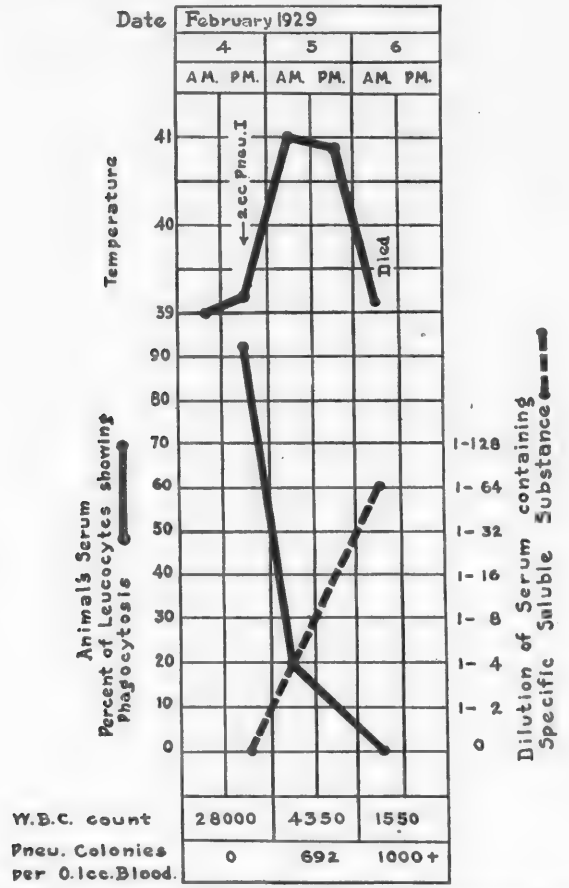

FIGURE III. Dog No. 2. Experimental pneumococcus infection following the intratracheal injection of 2 cc. Type I pneumococcus culture.

The results are shown in Figure III. At the end of 18 hours the opsonins were reduced but there were still enough present to cause a

slight degree of phagocytosis although approximately 6000 pneumococci per cc. were circulating in the blood stream and a demonstrable quantity of specific soluble substance was present in the serum. At the end of 42 hours the opsonins had disappeared completely while the number of pneumococci and amount of soluble substance in the serum had increased. The leucocyte count was reduced from 28,000 before infection to 1,550 on the day of death.

These three experiments, with others that followed a similar course, show that with an overwhelming pneumococcus infection accompanied by early blood invasion there is a rapid decrease in the concentration of humoral immune bodies which disappear entirely by the time of death. In other experiments in which animals recovered after a moderately severe generalized infection this same early diminution of humoral immune substances was observed to occur but the concentration of immune bodies began to rise with the onset of recovery which usually occurred about the fourth day. These immune bodies, appearing in the blood at the time of recovery, are of the so called acquired type as shown by Robertson and his co-workers (13).

In order to determine the nature of this decrease in humoral immune substances in a severe infection, a dog was injected with the filtrate of a 24 hour culture of pneumococcus. According to Avery and Heidelberger (14) the filtrate at this stage contains very little of the nucleoproteins of the pneumococcus but a considerable amount of the specific soluble substance which they have shown to be a carbohydrate, possibly a polysaccharide derived from the capsule of the cell. The experiment follows:

Experiment IV: Dog No. 3, weighing 16 kg., was bled 15 cc. from the heart and 50 cc. of filtrate from a 24 hour Type I pneumococcus culture were injected intravenously at 2 P.M. At 3 P.M. a sample of blood was drawn. At 4 P.M. 100 cc. of filtrate were injected intraperitoneally. Blood samples were drawn at 18 and 40 hours after injection.

The results are shown in Table II. There was a decrease in the opsonic action in the sample drawn one hour after injection. After 18 hours there was a slight return toward the initial opsonic titer but it did not return to normal until 40 hours after the filtrate injection. This diminution in opsonic activity is presumably due to the combina-

tion of the specific soluble substance with the natural immune bodies. Sia (15) using normal serum-leucocyte mixtures found that the presence of a very small amount of the purified soluble substance of the homologous type markedly altered the conditions in the mixture so that even a small number of avirulent pneumococci were enabled to grow in the serum and leucocytes of animals which possess the power to destroy ordinarily such pneumococci in relatively large numbers.

The opsonins in these experiments were highly type specific. The blood of animals infected with Type I pneumococcus did not show a decrease in opsonins when tested with a Type II organism and vice versa. The filtrate injections caused only a decrease in the opsonins for that specific type.

TABLE II

Effect of Intravenous Injection of Culture Filtrate on Opsonic Properties of the Serum

Kind of sensitizing serum	Degree of phagocytosis	
	Per cent of leucocytes showing phag.	Per cent showing 5 pairs or more
Serum before injection	78	66
One hour after injection	37	7
18 hours after injection	59	29
48 hours after injection	75	52

There has been evidence which has led some investigators (8) to think that a septicemia occurs, not because the natural immune substances have become exhausted, but because the organisms have become adapted to their host and have increased in virulence. In several dogs pneumococci were isolated from the blood stream at different stages of the septicemia and their susceptibility to phagocytosis compared with that of the organism originally used. No differences between them were found.

Localized Pneumococcus Infection

(*Lobar Pneumonia*)

A generalized and overwhelming infection as in the experiments above described is not the type of infection found characteristically

in lobar pneumonia. Although there may be a transient bacteremia and in some fatal cases a persistent bacteremia, lobar pneumonia, typically, is a localized pneumonic process. Lamar and Meltzer (16) produced with varying success, a lobar consolidation in dogs by injecting the pneumococcus culture deep into a bronchus by using a tracheal catheter. As a rule the dogs either developed a very light infection or the infection terminated fatally with empyema and a septicemia. With this in mind we attempted to produce a severe infection, yet an infection localized in the lungs. A similar method was employed except that the pneumococci were first suspended in a viscous medium (16 per cent gelatin broth). Because of the high degree of virulence for dogs, from 1 to 3 cc. of culture were sufficient to produce an infection. Five dogs out of nine treated in this manner developed a localized pneumonic infection. The other four died from septicemia and empyema.

Experiment V: Dog No. 4, weighing 17.6 kg., was bled 15 cc. from the jugular vein then etherized and a number 8 tracheal catheter was inserted as far as possible into a bronchus. Type I pneumococci from 3 cc. of an 18 hour culture were suspended in 16% gelatin broth solution and injected into a bronchus through the catheter. The next morning the dog appeared ill. The temperature had risen from 38.5°C. to 39.9°C. There was a marked cough, forced expiration, and an increased respiratory rate. X-ray of the chest taken on the third day showed consolidation of the three right lobes and the upper left lobe. The condition of the dog became progressively worse until death occurred on the sixth day. At autopsy the pleural cavities contained no fluid. The right lung was consolidated except for the lower half of the lower lobe which was air-containing. The left upper lobe was congested but air-containing. The left lower lobe appeared normal. The consolidated lobes were comparatively firm and gray in appearance. There was no fibrinous exudate covering the surface. On the cut surface was a grayish thick exudate. Microscopical sections revealed many polymorphonuclear leucocytes within the alveoli. There was only a slight amount of fibrin present. The heart's blood was negative for gram positive diplococci.

The results of this experiment are shown in Figure IV. The most important observation made was the fact the dog died with a negative blood culture. There was an early blood invasion noted 18 hours after infection but the remaining blood cultures proved negative. The pneumococcidal promoting power of the blood did not diminish to

an appreciable extent throughout the course of the infection. The opsonic properties of the serum corresponded in general with the pneumococcidal titers. Evidently the humoral immune substances in

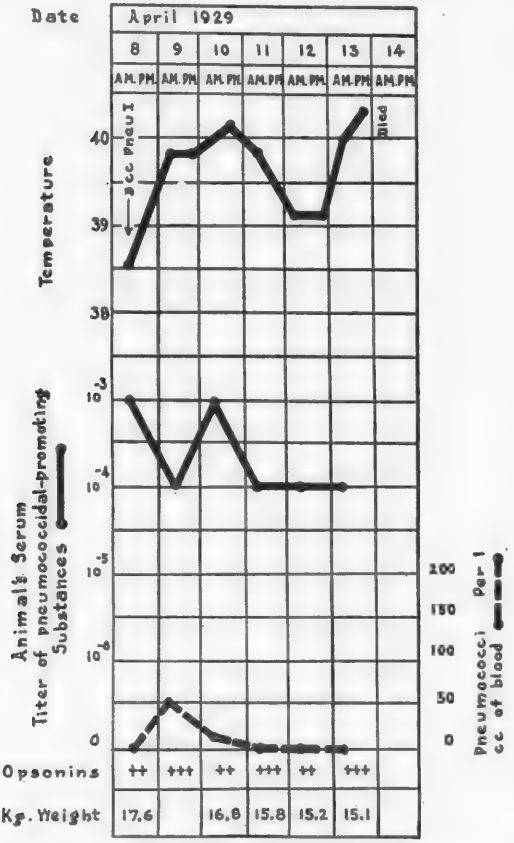

FIGURE IV. Dog No. 4. Experimental pneumococcus infection following the intrabronchial injection of 3 cc. Type I pneumococcus culture.

this case were sufficient to prevent invasion of the blood stream but, on the other hand, did not prevent the spread of the infection within the lung which was lobar in distribution.

Experiment VI: Dog No. 5, weighing 16 kg., was bled 15 cc. from the jugular vein, etherized, and pneumococci Type I from 2 cc. of culture were suspended in 16% gelatin broth solution and injected deep into a bronchus by intrabronchial insufflation. The next morning the dog appeared quiet, would not eat, and its

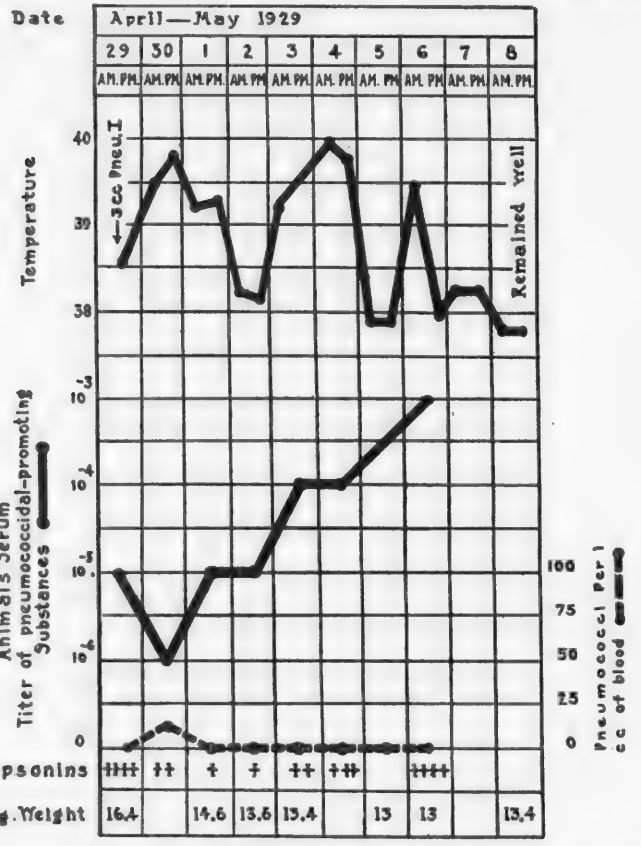

FIGURE V. Dog No. 5. Experimental pneumococcus infection following the intrabronchial injection of 3 cc. Type I pneumococcus culture.

temperature was slightly elevated. X-ray showed marked consolidation of the right lower and middle lobes. The third day the respiration had increased to 92 per minute, there was marked dullness on the right side to percussion, and on auscultation marked bronchial breathing was heard on the same side. On the seventh day the temperature subsided and the condition of the dog improved.

As in the previous case, there was a slight early blood invasion which did not persist after the second day (Figure V). The pneumococcidal promoting action of the serum was slightly decreased after 18 hours but there was still an appreciable degree present. On the third day the titer of this serum property had returned to normal and showed a further increase as long as the blood specimens were taken. Tests for opsonic activity showed a more marked decrease in this property but it followed a similar course.

Experiment VII: Dog No. 6, weighing 22 kg., was bled 15 cc. from the jugular vein, anesthetized with ether, and 3 cc. of Type I pneumococcus suspension injected deep into a bronchus. The next morning the dog appeared quite sick. The temperature was elevated. X-ray revealed a consolidation of the right lower lobe. The condition remained about the same until the fourth day when signs of fluid developed in the right chest. The dog died on the seventh day. Autopsy revealed a thick grayish fluid in the pleural cavity of each side. The pericardial cavity contained about 75 cc. of like material. Stained smear showed innumerable gram positive diplococci. The lobes of the right lung were well collapsed and dark red in color. The left lower lobe was congested but air-containing. The left upper lobe appeared normal. No true consolidation was seen. Microscopical sections from the lobes of the right lung showed congestion of the alveolar walls and red cells in some of the alveolar spaces. Very few leucocytes were seen.

The humoral immune changes occurring in this dog differed considerably from those in the preceding animals. 18 hours after infection the immune substances had not decreased (Figure VI) although there were 86 pneumococci per cc. in the blood. After 42 hours however, there was a marked reduction in the pneumococcidal-promoting substances and a slight diminution in the number of organisms per cc. of blood. By the fourth day of the disease the concentration of immune bodies had shown a rise while the number of circulating pneumococci had fallen to only 16 per cc. of blood. The following day when there were definite signs of fluid in the chest, the titer of the pneumococcidal-promoting substances was back to normal but there was a marked increase in the bacteremia; 280 organisms per cc. of blood were noted. The bacteremia increased until death although the pneumococcidal-promoting substances were still present.

In attempting to correlate the findings in this animal with the preceding observations, account must be taken of the type of lesion pres-

ent in the lung. Judging X-rays taken 24 hours after the onset of the infection there was a definite beginning localization of the pneu-

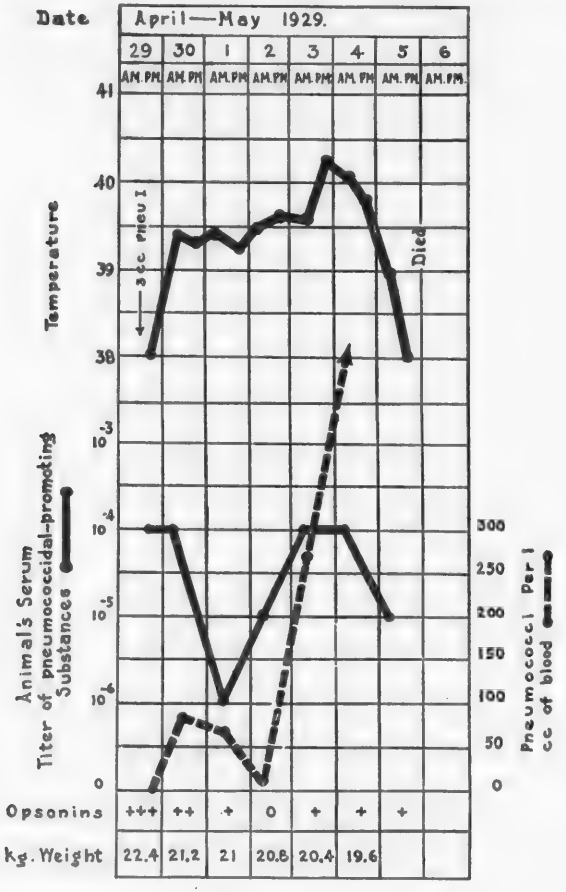

FIGURE VI. Dog No. 6. Experimental pneumococcus infection following intrabronchial injection of 3 cc. Type I pneumococcus culture.

monic process. While blood invasion at this stage was somewhat more marked than in dogs (3) (4), it was by no means as pronounced as in those animals suffering from a generalized pneumococcus infection.

As in the other dogs with lobar pneumonia this initial blood invasion diminished but not as quickly nor did the pneumococci disappear from the blood completely. From this it could be inferred that localization of the lung process did not develop as in the dogs with true lobar pneumonia. The lack of definite pneumonic consolidation found at autopsy bore out this assumption. The terminal state of the blood in which large numbers of pneumococci were circulating in the presence of a considerable concentration of pneumococcidal-promoting substances is not susceptible to a satisfactory explanation on the basis of the data available. It would seem most probable, however, that the phagocytic cells of the body were failing to function adequately. While leucocyte counts were not made in this animal, observations on other dogs with overwhelming pneumococcus infection showed a great reduction in the number of circulating leucocytes. The onset of empyema may have produced a depression of functional activity of the phagocytic cells of the body as well. The excess of antibody over antigen might be accounted for by the fact that at this stage of the disease, 4th to 5th day, the immune substances present were of the acquired type and being produced in a concentration much greater than that of the normal antibodies and yet one would expect that if the immune substances were active the pneumococci would be agglutinated and swept out of the blood stream as shown by Bull (8). An alternative possibility could be the presence of some unknown factor operating to prevent the union of antigen with antibody. That the invading pneumococcus had become resistant to the dog's immune bodies seems unlikely in view of studies on this point in other animals.

SUMMARY

A study was made of the changes in humoral immunity occurring during the early phases of experimental pneumococcus infection in the dog and cat. The methods devised by Robertson and Sia were employed to demonstrate the presence of anti-pneumococcus properties in the serum of animals naturally resistant to this micro-organism. It was found that with a generalized and overwhelming infection accompanied by early blood invasion, there was a prompt and rapid decrease in the concentration of natural humoral immune bodies which frequently disappeared entirely by the time of death. This same

early diminution of humoral immune substances, opsonins, agglutinins, and pneumococcidal-promoting bodies was observed in animals that survived a moderately severe generalized infection but the concentration of immune bodies rose again with the onset of recovery. The decrease in concentration of humoral immune substances during a severe generalized infection appeared to be due to the combination of "S" substance with the normal immune bodies.

When the pneumococcus infection was more localized as in the case of true lobar pneumonia a quite different sequence of events was observed to occur. Several animals, in which extensive lobar pneumonia was produced, showed the presence in quantity of humoral immune bodies in the blood throughout the course of an infection terminating fatally.

These findings suggest that after the inception of pneumococcus infection in the dog and cat the chief function of natural anti-pneumococcus substances in the blood is to limit or prevent blood invasion. When pneumococcic infection is localized these circulating antibodies appear to have little effect either in preventing the spread of the process or determining the outcome of the disease.

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REACTIONS OF RABBITS TO INTRACUTANEOUS INJECTIONS OF PNEUMOCOCCI AND THEIR PRODUCTS

I. The Antibody Response

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(Received for publication, December 31, 1929)

During the course of studies on the effectiveness of Pneumococcus antigen under different conditions, unexpected results were observed following the introduction of the bacteria into the skin. It was found that the antibody response under this condition is strikingly different from that which is evoked by intravenous injection. The response of the animal to the intracutaneous injection of pneumococci has been subjected, therefore, to a thorough study. Some of the results have been stated in a preliminary communication (1). Further work is reported here.

Methods

1. Preparation of Vaccines. Eight to ten hour broth cultures of Pneumococcus were centrifuged at a high rate of speed, the supernatant fluid was discarded, and the organisms were suspended in an amount of saline solution equal to one-tenth of the original volume of culture. Occasionally, the sedimented bacteria were washed 1 to 3 times before final resuspension. The concentrated emulsion of bacteria was then heated for one hour at 56–60°C. and sterility was determined by cultures and intraperitoneal injection of white mice.

2. Preparation of the Skin. In the earlier experiments the fur was removed by clipping and shaving, but later this was accomplished by gentle rubbing with a saturated solution of barium sulfite. The latter method proved more satisfactory, since, with care, it left a perfectly clear skin which remained free of fur for a number of weeks. The depilation was carried out on both sides of the body, and, since consecutive injections were made, it frequently became necessary to repeat the removal of fur.

3. Method of Injection. The suspensions of heat-killed pneumococci were injected intracutaneously once a week in quantities of 0.2 cc. This amount was equivalent to 2.0 cc. of the original culture. The injections were given alternately on the two sides of the body, each injection being made in a different area of the skin. The

number of injections in the different animals varied. In the majority of instances, the total amount of suspension given was equivalent to 20 cc., or more, of culture. The results obtained, therefore, could be roughly compared with those observed following intravenous immunization of rabbits with similar amounts of culture.

4. Testing for Antibodies. Some of the animals were tested for antibody production only after a complete series of ten injections and others at different intervals during the process. Usually the rabbits were bled once a week, immediately preceding each injection. Final specimens of blood were procured 10 days following the last injection. The sera were tested for the presence of type-specific antibodies (anti-S), which agglutinate the encapsulated, or S, cells and precipitate the soluble specific substance derived from them, and also for species-specific antibodies (anti-P or anti-R) which agglutinate only the capsule-free, or R, cells and precipitate the protein derived from any type of Pneumococcus.

EXPERIMENTAL

Rabbits were injected into the skin with suspensions of heat-killed Pneumococcus Type I or Type III, or an R strain derived from Type II Pneumococcus. In addition, intracutaneous injections were made with solutions of derivatives of Pneumococcus, one of which consisted essentially of a solution of "nucleoprotein" (2) and the other of the supernatant fluid after acid precipitation of the "nucleoprotein" from a solution of pneumococci resulting from repeated freezing and thawing. The strain of Type I Pneumococcus employed was virulent for white mice and rabbits, and killed animals of both species when 10^{-7} cc. of culture was administered. The Type III culture killed mice in a quantity of 10^{-7} cc. of culture, but did not kill rabbits in amounts as high as several cc., although in these animals it was capable of producing a transient septicemia. In one experiment a rabbit-virulent strain of Type III Pneumococcus was used as antigen. The lethal dose of this strain varied from 10^{-3} to 10^{-4} cc. of culture.

Antibody Response to Type I (S) Pneumococcus

Several groups of rabbits (sixty animals in all) were given repeated intracutaneous injections of suspensions Pneumococcus Type I. The development of antibodies was essentially the same in all the animals so that for the sake of brevity and clarity, one group will be described in detail as typical. The data obtained in the study of this group are represented graphically in Figure 1. The curves for the development of type-specific and species-specific agglutinins were plotted from the averages of the titres of the sera of a group of four rabbits. These rabbits had received 12 intracutaneous injections of 0.2 cc. each of a suspension of heat-killed pneumococci of Type I (S form), a total number of bacteria equivalent to those contained in 24 cc. of culture,

which is more than the amount required for the usual intravenous immunization. The astonishing result was the absence of type-specific agglutinins and type-specific precipitins in the sera of all the animals. In other words, the sera did not cause the agglutination of the bacteria employed in the immunization, nor precipitate the soluble specific substance derived from them. Moreover, this result was obtained not only in this particular group of animals, but in a total of 60 rabbits receiving repeated intracutaneous inoculations of Type I Pneumococcus only 7, or about 12 per cent, showed any evidence of type-specific response, and in these instances specific agglutinins were demonstrable only in low dilutions of the serum. In two

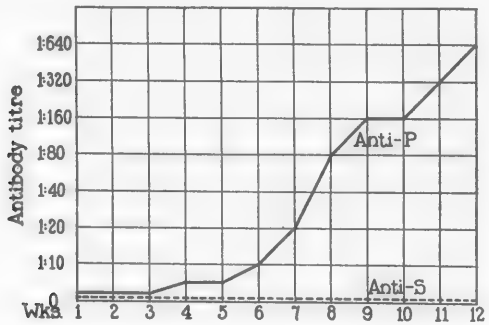

FIGURE 1. The development of antibodies in rabbits immunized intracutaneously to Pneumococcus, Type I.

instances agglutination occurred in a dilution of 1:1, in one instance 1:3, in three instances 1:5, and in one case 1:20. Obviously, these results are in marked contrast to those obtained with the sera of animals which have received similar quantities of organisms administered intravenously, in which case agglutination usually occurs in dilutions of 1:80 to 1:160. The lack of type-specific antibodies can be interpreted as evidence of the disintegration of the type-specific antigen after the introduction of the intact cell into the skin.

Despite the fact that type-specific (anti-S) antibodies were absent, the species-specific antibodies (anti-P or anti-R) were found to be present in high degree in the sera of all the rabbits. As illustrated in

Figure 1 the species-specific antibodies, as measured by the agglutination of R cells, are definitely detectable after the third or fourth injection, and after about the sixth injection there is a constant and rapid rise in titre which may reach a serum dilution of 1:640. In the sera of some rabbits, not included in the accompanying curves, the concentration of species-specific antibodies was even higher.

Antibody Response to Type III (S) Pneumococcus

In terms of its capacity to stimulate the formation of type-specific antibodies, Type III Pneumococcus is at best a poor antigen. The recent work of Tillett (4) has established the fact that Type III strains

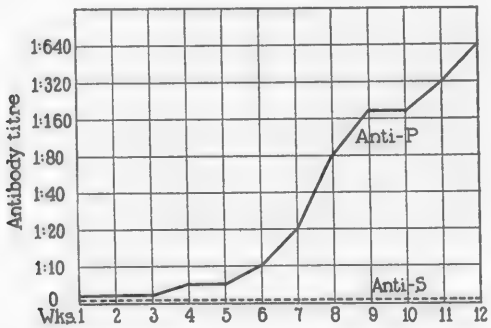

FIGURE 2. The development of antibodies in rabbits immunized intracutaneously to Pneumococcus, Type III.

of marked virulence for mice are usually not only avirulent for rabbits, but in only a small percentage of rabbits (15 per cent) do they elicit a type-specific antigenic response following intravenous inoculation. Moreover, his results indicate that with Type III Pneumococcus there is a parallelism between type-specific antigenicity and virulence. It was to be expected, then, that rabbits immunized intracutaneously with Type III pneumococci would respond very poorly in the production of type-specific antibodies.

In Figure 2, curves are given which illustrate the development of anti-S and anti-R agglutinins. The rabbits were given 12 injections of 0.2 cc. each of a suspension of Type III pneumococci, thus receiving

in all the equivalent of 24 cc. of culture. It will be seen that at no time were type-specific antibodies demonstrable either as agglutinins or precipitins. None of a total of 45 rabbits, studied at different times, possessed anti-S antibodies in their sera during or following repeated intracutaneous injections of Type III pneumococci.

On the other hand, in the sera of all the rabbits species-specific antibodies were demonstrable. As Figure 2 illustrates, the development of anti-R agglutinins in these rabbits closely parallels that observed in the group immunized intracutaneously to suspensions of Type I pneumococci.

Antibody Response to R Pneumococcus

That R organisms fail to incite the formation of type-specific antibodies has been the common experience of all workers. The intravenous administration of R cells leads only to the development of species-specific antibodies. In studying the antibody response in rabbits following intracutaneous injections, an R strain derived from Type II was employed as antigen, and in each instance the total amount of heat-killed suspension employed was equivalent to at least 20 cc. of culture. None of the rabbits immunized in this manner formed type-specific antibodies. The serum of all the rabbits, however, contained species-specific agglutinins and precipitins in titres comparable to those observed in rabbits immunized to Pneumococcus Type I and Type III.

The injection into the skin of the Pneumococcus cell, whether R or S, elicits an antibody response which is essentially or entirely of the species-specific variety. It seems likely, therefore, that the type specificity of the S form is destroyed when it is introduced into the skin.

Antibody Response to Soluble Derivatives of Pneumococcus

In the preceding experiments rabbits were immunized by the inoculation of the intact, formed cells. The studies of Reimann (5) have revealed that the antigenic response of animals to injection of the "nucleoprotein" of Pneumococcus is identical with the response to the intact R cells. It was interesting, therefore, to compare the antigenic response of rabbits to the intracutaneous injections of soluble derivatives of Pneumococcus with the response to the similar injection

of the intact cells. The soluble derivatives were obtained from an S strain of Type II Pneumococcus. The cells were frozen and thawed repeatedly, and from the resulting solution of bacterial substance, by precipitation with acetic acid in the cold, a material designated for convenience as "nucleoprotein" (2) was obtained. The residual supernatant fluid, after precipitation with acetic acid, was also studied. This supernatant fluid contains, in addition to other substances, the purpura producing material of Pneumococcus (3, 6). The two soluble derivatives were inoculated consecutively once a week into the skin of rabbits in a volume of 0.2 cc. each. The substances were standardized on the basis of protein nitrogen, and rabbits received in toto at least 70 mgm. of the first substance and at least 30 mgm. of the latter considered as protein.

As was to be expected, none of the rabbits injected with either of these two soluble derivatives showed anti-S in their sera. This is in agreement with previous results (7) from this laboratory. On the other hand, all of the rabbits possessed precipitins for the "nucleoprotein" and agglutinins for the R cells but in somewhat lower titre than that demonstrated in the sera of rabbits immunized by either the intact R or S forms of Pneumococcus.

Antibody Response to Intravenous Injections after Previous Intracutaneous Immunization

The marked differences in antibody responses to type-specific antigens, depending upon whether the intravenous or intracutaneous route of administration was employed, raised the question whether the property of inducing disintegration or dissociation of the type-specific antigen is possessed only by certain tissues, such as the skin, or whether all tissues of the animal body are potentially able to induce this disintegration, and whether, as a result of repeated intracutaneous injections, this property might be so exalted that subsequent intravenous introduction of the bacteria would fail to elicit a type-specific antibody response. In order to determine the answer to this question the following experiment was made. Twelve rabbits were selected which, following repeated intracutaneous immunization, did not show type-specific antibodies in their sera. Six had been immunized with a suspension of heat-killed Type I pneumococci, and six with a suspension

of Type III pneumococci. All twelve were then immunized by intravenous injections of a suspension of heat-killed Type I pneumococci. Following the intravenous immunization, the sera of all 12 rabbits were found to agglutinate Type I cells specifically. The titre in each case reached as high as 1:160. This titre is slightly higher than that usually found in normal rabbits after intravenous immunization alone.

DISCUSSION

The significant result of the present study is that the type-specific antigen of pneumococcus does not stimulate the production of antibodies when it is injected into the skin. This is in marked contrast with the results obtained when the injections are made intravenously. Following intracutaneous injections of the whole organisms the resulting antibodies are of the species-specific type. While in the skin the type-specific substances of the bacterial cell, therefore, become ineffective, the remaining constituents of the pneumococcal cell retain the power of stimulating antibody formation. The antibody response to intracutaneous inoculations is the same whether the type-specific cells (S forms), the cells which do not possess type-specific characters (R forms), or the soluble protein constituents of the cell are injected.

Why the type specific antigen of the cell, when injected into the skin, becomes ineffective in stimulating antibody response is not entirely clear. It is possible that in the skin the bacteria are mechanically localized and that, as a result, the cells undergo a disintegration, comparable to autolysis, during which the type-specific antigen is destroyed. It is more likely that the skin possesses a peculiar property of causing disintegration or dissociation of the compound type-specific antigen, so that the latter is no longer able to stimulate the production of antibodies.

Similar failure of pneumococci to stimulate the production of type-specific antibodies when the bacteria are injected into the skin has been noted by others. Goodner (8) found that when live virulent cultures of Type I pneumococci were injected into the skin of rabbits half of the animals failed to develop type-specific antibodies in their sera. Gross (9) has also found that following the intracutaneous injections of small amounts of live pneumococci in rabbits, no agglutinins appeared in the sera. Bull and McKee (10) found that type-specific antibodies

frequently failed to appear in the sera of animals after pneumococci had been instilled into the nose. It is of much significance that in the experiments of these three sets of observers antibodies failed to develop following the injection of live virulent pneumococci, in two cases into the skin, and in the other case into the nose.

SUMMARY AND CONCLUSIONS

1. Sixty rabbits were immunized by the repeated injections into the skin of small doses of suspensions of heat-killed Type I pneumococci. In 53 of the rabbits no type-specific antibodies appeared in the serum, and in the remaining seven the titre of these antibodies in the serum was very low. In all cases, however, the sera possessed a high titre of species-specific antibodies.

2. Forty-five rabbits similarly immunized by injections of heat-killed Type III pneumococci also failed to form type-specific antibodies but did form species-specific antibodies.

3. Suspensions of heat-killed R pneumococci and solutions of bacterial substances when injected into the skin stimulated the production of species-specific antibodies, although they failed to stimulate the production of any type-specific antibodies.

4. Animals which had been immunized by intracutaneous injections still possessed the ability to form type-specific antibodies when they were subsequently given intravenous inoculations of type-specific pneumococci.

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REACTIONS OF RABBITS TO INTRACUTANEOUS INJECTIONS OF PNEUMOCOCCI AND THEIR PRODUCTS

II. Resistance to Infection

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The results reported in the preceding communication (1) disclose the fact that the antibody responses to the injection of heat-killed pneumococci differ qualitatively, and that the nature of the response is related to the route of administration of the organisms. Pneumococci of Type I, which are invariably effective in stimulating the formation of type-specific antibodies when they are introduced intravenously, were ineffective in producing this type of antibody response in 88 per cent of the rabbits when the injections were made intracutaneously. Under the latter conditions, the antibody response was solely or predominantly species-specific and was no different from the response of rabbits to the intracutaneous injection of S cells of different types, or, indeed, of degraded, non-type-specific R organisms.

The question naturally suggests itself whether rabbits which have received repeated intracutaneous injections of heat-killed pneumococci, and therefore have in their sera no type-specific antibodies, are, nevertheless, actively resistant to infection, and if so, whether this form of acquired resistance is likewise effective against infection by heterologous types of Pneumococcus. The question also arises whether the sera of animals immunized by intracutaneous injections will confer passive protection upon white mice against infection by homologous types of Pneumococcus. A preliminary report (2) has already been made of the experiments here presented which were undertaken to solve these problems.

EXPERIMENTAL

Rabbits were immunized by repeated intracutaneous injections. The inoculations were made once a week and consisted of 0.2 cc. of a suspension of heat-killed

bacteria, or the equivalent of 2.0 cc. of broth culture. The degree of resistance to infection was determined 10 to 21 days after the last intracutaneous inoculation by injecting intravenously varying quantities of live virulent cultures of Type I or Type III pneumococci. The Type I strain killed normal rabbits in amounts of 10^{-7} cc. of broth culture, while the Type III strain was fatal in an amount of 10^{-3} cc. to 10^{-4} cc. Samples of blood were obtained from the rabbits immediately preceding infection and the sera were used to determine the titre of type-specific agglutinins and also the power to protect white mice against infection by pneumococci of homologous types.

Resistance to Infection Following Intracutaneous Immunization with Heat-Killed "S" Cells of Pneumococcus

I. Immunization with Type I (S) Pneumococcus

Forty rabbits were immunized intracutaneously to Type I Pneumococcus and tested later for their resistance to infection. In the earlier experiments the resistance to infection by the homologous type of Pneumococcus was determined, while in the later experiments the degree of active immunity to infection by some type of Pneumococcus other than that used for immunization was tested.

(*a*) *Active Resistance to Infection by Pneumococcus Type I (Homologous).* Resistance to infection by the homologous type of Pneumococcus was first determined in a group of three rabbits (Table I).

Each had received into the skin 12 injections of suspensions of heat-killed pneumococci, the total number of bacteria injected being equivalent to those contained in 27 cc. of broth culture. Twelve days after the last inoculation all the animals were given intravenously 0.1 cc. of living virulent Type I culture, and all survived. Control, normal rabbits died within 48 hours after receiving 0.0,000,001 cc. of the same culture. The serum of one of the 3 immunized rabbits possessed an agglutinin titre for the type-specific organism of 1:1; sera from the other two rabbits had no agglutinating power. A second group of 9 rabbits received 8 injections of heat-killed pneumococci equivalent to those contained in 16 cc. of broth culture. Subsequently they survived the intravenous injection of live cultures in quantities as large as 0.2 cc., whereas normal rabbits succumbed following injections of 0.0,000,001 cc. In only 3 of the rabbits were type-specific agglutinins demonstrable.

These observations demonstrate that intracutaneous immunization with heat-killed Type I pneumococci is followed by resistance to the intravenous injection of virulent cultures of the same type.

(*b*) *Active Resistance to Infection by Type III Pneumococci (Heterol-*

ogous). The foregoing experiment shows that rabbits may acquire an effective resistance to infection by the same organism used in immunization, even when demonstrable circulating type-specific antibodies are absent. Consequently it was interesting to determine whether this form of resistance is also effective against infection with pneumococci of heterologous types. Accordingly, in all the following experiments resistance was determined to infection by Pneumococcus Type III. In Table I are given results of these experiments in 31 rabbits. It is evident that following intracutaneous administration of suspensions of heat-killed Type I pneumococci, rabbits acquire a marked degree of resistance to infection by virulent Type III organisms. In a general way, the resistance increases with the number of injections. However, in the last experiment given in Table I the rabbits received only one injection and yet they were resistant to 100 lethal doses of Pneumococcus Type III.

(*c*) *Passive Immunity.* It is seen, then, that rabbits may be actively immunized by intracutaneous injections, not only against pneumococci of the type used in immunization but also against pneumcococci of other types, and this in the absence of circulating type-specific antibodies. It was interesting to determine whether the sera of these rabbits protected white mice against infection. Protection tests were conducted in the usual manner by the simultaneous intraperitoneal injection of 0.2 cc. of serum and varying dilutions of Type I Pneumococcus culture. The sera of 53 animals were tested (Tables I and III); the sera of 42 of them showed no measurable protective properties for mice, while the sera of the remaining 11 exhibited protective power against only very small infecting doses of Pneumococcus Type I culture. Of the 11 sera which were protective, 6 contained no type-specific agglutinins while 5 showed type-specific agglutinin titres of 1:1–1:20. On the other hand, of the 42 sera with no protective capacity only one showed type-specific agglutinins, and in this case the agglutination titre was 1:3. It is evident, therefore, that in most instances in which the sera possessed protective power this was associated with the presence of type-specific agglutinins. On the other hand, in most of the animals the sera possessed no protective power for mice and no type-specific agglutinins. It is possible, however, as the work of Tillett (3) suggests, that these sera might have protective power for rabbits,

TABLE I

Resistance of Rabbits to Infection Following Intracutaneous Immunization with Heat-Killed Pneumococcus, Type I

Number of rabbits	Number of injections	Total amount injected	Immunity to Type I (homologous)			Immunity to Type III (heterologous)			Protective capacity for mice		Anti-S titre
			cc.	Survived	Died	cc.	Survived	Died	Number of sera	Degree of protection	
		cc.									
									2	none	negative
3	12	27	0.1	3	—	—	—	—	1	10^{-5}	1–1:1
									1	10^{-2}	1:20
9*	8	16	0.2	3	—	1.0	1	1	1	10^{-3}	1:1
									2	10^{-4}	1:5
			0.1	3	—	0.8	2	1	2	10^{-5}	negative
									1	10^{-6}	negative
			0.01	3	—	0.5	3	—	2	none	negative
5	7	14	—	—	—	—	—	—	5	none	negative
4	14	29	—	—	—	1.0	2	—	4	none	negative
						0.5	2	—			
5	10	20	—	—	—	1.0	—	2			
						0.8	—	2	5	none	negative
						0.5	1	—			
2	5	10	—	—	—	1.0	1	—			
						0.5	1	—	2	none	negative
2	10	20	—	—	—	1.0	1	—	1	10^{-6}	negative
						0.5	1	—	1	none	negative
9	1	2	—	—	—	0.5	—	1			
						0.3	—	2	9	none	negative
						0.1	—	2			
						0.01	2	—			
						0.001	2	—			

M. L. D. of Type I culture for normal rabbits, 10^{-7} cc.; for mice 10^{-7} cc.

M. L. D. of Type III culture for normal rabbits, 10^{-4} cc.; for mice 10^{-7} cc.

* In this instance, all nine rabbits were tested first for resistance to Type I infection; and later to Type III.

but this question has not been studied. He observed that, following intravenous immunization with R pneumococci, rabbits not only acquired an active resistance but that the sera of these animals, passively transferred to other rabbits, protected them against infection with pneumococci of every type. These sera, however, afforded no protection to mice against pneumococcal infection.

II. Immunization with Type III (S) Pneumococcus

Thirty-eight rabbits were immunized by intracutaneous injections of heat-killed Type III pneumococci (Table II). These animals were subsequently tested for active immunity to virulent cultures of Type I or Type III Pneumococcus.

(*a*) *Active Immunity to Infection by Type III (Homologous) Pneumococcus.* Resistance to infection by pneumococci of the homologous type was tested in 9 rabbits.

They were immunized by 8 injections into the skin of heat-killed pneumococci Type III, the total number of bacteria corresponding to those contained in 16 cc. of broth culture. All the animals were subsequently infected by intravenous injections of varying amounts of culture in quantities as large as 1.0 cc. The culture employed killed normal rabbits in doses of 0.0001 cc. One of the animals died and 2 survived after receiving 0.5 cc. of culture, 2 died and 1 survived after 0.8 cc., and 1 died and 2 survived after 1.0 cc. of culture. In the 4 animals which succumbed, death was delayed from the seventh to the tenth day after the infection, and in each instance, the post-mortem examination revealed a massive pericarditis and pleurisy. The delayed death and localization of the infection may be considered to be an expression of active immunity.

It is obvious that these animals showed a considerable degree of active immunity to infection with homologous organisms.

(*b*) *Active Immunity to Infection by Type I Pneumococcus (Heterologous).* Twenty-eight rabbits which had been immunized by the intracutaneous injection of heat-killed pneumococci (Type III) were later tested for their immunity against infection with heterologous (Type I) pneumococci (Table II). The culture employed in the tests was of such a virulence that 0.0,000,001 cc. injected intravenously regularly killed normal rabbits. The immunized animals received doses of from 0.001 to 0.2 cc. Of the 28 immunized animals receiving these large doses, 22 recovered and only 6 died. It is evident, therefore, that the animals immunized by intracutaneous injections had

acquired a marked resistance against pneumoccci of a heterologous type.

(*c*) *Passive Immunity.* It is well known that only in rare instances is the serum of a rabbit immunized intravenously to Type III Pneu-

TABLE II

Resistance of Rabbits to Infection Following Intracutaneous Immunization with Heat-Killed Pneumococcus, Type III

Number of rabbits	Number of injections	Total amount injected	Immunity to Type III (homologous)			Immunity to Type I (heterologous)			Protective capacity for mice		Anti-S titre
			cc.	Survived	Died	cc.	Survived	Died	Number of sera	Degree of protection	
		cc.									
9	8	16	1.0	2	1						
			0.8	1	2				9	none	negative
			0.5	2	1						
3	12	27	—	—	—	0.01	3	—	3	none	negative
5	7	14	—	—	—	—	—	—	5	none	negative
4	14	29	—	—	—	0.1	1	1			
						0.01	2	—	4	none	negative
5	10	20	—	—	—	0.2	—	1			
						0.1	1	—	5	none	negative
						0.01	1	1			
6	7	14	—	—	—	0.1	1	1			
						0.01	2	—	—	—	—
						0.001	2	—			
5	7	14	—	—	—	0.1	2	—			
						0.01	1	1	5	none	negative
						0.001	1	—			

mococcus protective for white mice against infection with homologous organisms. In the experiments here recorded (Table II) the sera of 37 rabbits which were immunized intracutaneously with suspensions of heat-killed pneumococci Type III were tested for their protective power in white mice, and in no instance did these sera confer any

measurable protection. Moreover, in none of the sera were any type-specific antibodies demonstrable.

According to the recent work of Sia (4) normal pig serum, though lacking type-specific agglutinins, protects mice against infection by Pneumococcus of any type. The special technique which he employed was used in retesting six of the sera mentioned above. Three of these sera were from the animals immunized by the injection of Type III pneumococci and three from the rabbits immunized with Type I pneumococci. The serum was injected intraperitoneally into the mice 4 hours preceding the inoculation of the live culture. No protective power could be demonstrated in any of these sera by this method.

The Rate of Development and Duration of Active Immunity Following Intracutaneous Injections

Having determined that rabbits, following repeated intracutaneous immunization, acquire a marked resistance to infection by Pneumococcus of any type, observations were next made to determine how rapidly this form of active immunity develops and how long it persists.

Fourteen rabbits were immunized, employing a varying number of intracutaneous injections and different amounts of heat-killed bacteria (Table III (a)). Each of two animals received from 1 to 7 injections of the bacteria, corresponding to 2 cc. to 14 cc. of Type I pneumococcal culture. Three weeks after the last injection, the immunity to infection was measured against Type III pneumococci.

The results show that even after one intracutaneous injection of pneumococci, in an amount corresponding to 2.0 cc. of culture, rabbits may show a distinct resistance to infection with heterologous pneumococci. Of the 14 rabbits, the sera of only 2 showed any anti-S agglutinating power. In one rabbit, which had received 4 injections, the agglutination titre of the serum was 1:3, and in the other, which had received 7 injections, the titre was only 1:5. The protective action of these sera for mice was very low and irregular, only 3 sera affording minor protection against homologous infection. Curiously enough, the 2 sera possessing specific agglutinins afforded no protection to white mice.

Six rabbits of a second group (Table III (b)) were each immunized

TABLE III

Rate of Development and Duration of Immunity in Rabbits Immunized with Heat-Killed Pneumococcus Type I by the Intracutaneous Route

(*a*) *Rate of Development*

Number of rabbits	Number of injections	Total amount injected	Immunity to Type III (heterologous)			Protective capacity for mice		Anti-S
			cc.	Survived	Died	Number of sera	Degree of protection	
		cc.						
2	1	2	0.01	1	—			
			0.01	1	—	2	none	negative
2	2	4	0.1	1	—			
			0.3	1	—	2	none	negative
2	3	6	0.3	1	—			
			0.5	1	—	2	none	negative
2	4	8	0.5	1	—	1	none	negative
			0.5	1	—	1	none	1:3
2	5	10	0.5	1	—	1	none	negative
			0.8	1	—	1	10^{-5}	negative
2	6	12	0.8	1	—	1	10^{-6}	negative
			1.0	1	—	1	none	negative
2	7	14	0.5	—	1	1	none	negative
			1.0	1	—	1	none	1:5

(*b*) *Duration of Immunity*

Animals Immunized with Pneumococcus, Type III

Number of rabbits	Number of injections	Total amount injected	Immunity to Type I (heterologous) after 6 months			Protective capacity for mice	Anti-S
			cc.	Survived	Died		
		cc.					
1	8	16	10^{-1}	—	1	All sera negative both 10 days and 6 months after final injection	
1	8	16	10^{-2}	—	1		
1	8	16	10^{-3}	—	1		
1	8	16	10^{-4}	1	—		
1	8	16	10^{-5}	1	—		
1	8	16	10^{-6}	1	—		

by intracutaneous injections of a suspension of Type III organisms corresponding to a total amount of 16 cc. of culture. Six months after the final injection the animals were infected with Type I Pneumococcus culture in amounts varying from one-tenth to one-millionth cc. As shown in the table the animals were resistant to infection with one-ten-thousandth cc. It appears, therefore, that resistance to heterologous infection still persists after six months although at this time it is considerably diminished.

Resistance to Infection Following Intracuaneous Immunization with Suspensions of Heat-Killed R Cells of Pneumococcus

The R strain used for immunization was originally derived from a Type II Pneumococcus. Rabbits were immunized by intracutaneous injections of suspensions of heat-killed pneumococci in a manner similar to immunization with S cells. The animals received amounts of suspension equivalent to those usually employed in intravenous immunization. Resistance to infection was measured by injecting intravenously different quantities of a virulent culture of Type I pneumococci. The results given in Table IV show that 4 rabbits immunized to R pneumococci survived following the injection of 0.01 cc. or 0.1 cc. of a Type I Pneumococcus culture which killed normal rabbits in a dilution of 0.0,000,001 cc. The sera of the rabbits immunized with R organisms derived from Type II pneumococci afforded white mice no protection against infection by Type II pneumococci. The sera also did not agglutinate Type II pneumococci.

Resistance to Infection Following Intracutaneous Immunization with Soluble Derivatives of Pneumococcus

It has previously been shown that following intracutaneous immunization with S or R pneumococci, rabbits acquire an active immunity to infection by homologous and heterologous types of pneumococci. It was also shown in a previous paper (1) that the antibody response in the majority of rabbits is the same (species-specific) whether intracutaneous immunization is brought about by the injection of formed cells or by the injection of soluble derivatives of Pneumococcus. The present observations were made to determine whether, following intracutaneous injections of solutions containing soluble derivatives of

Pneumococcus, rabbits become actively immune to infection with living cultures.

The animals received (a) a solution of "nucleoprotein," and (b) the supernatant fluid after acid precipitation of the "nucleoprotein" from a solution of pneumococci resulting from repeated freezing and thawing. These solutions were prepared from a culture of Type II Pneumococcus in the same manner as described in the previous paper (1).

The rabbits receiving "nucleoprotein" were given repeated injections, the total amount of protein administered to each animal, as estimated by nitrogen deter-

TABLE IV

Resistance of Rabbits to Infection Following Intracutaneous Immunization with R Forms and with Soluble Protein Derivatives of the Cell

Antigen administered	Number of rabbits	Quantity of antigen	Immunity to Type I (heterologous)			Protection conferred by sera on mice (homologous infection)		
			cc.	Survived	Died	Number of sera	Degree of protection	Anti-S titre
R Pneumococcus Derived from Type II "S"	4	20 cc.	10^{-1}	2				
			10^{-2}	2		4	none	negative
Nucleoprotein Derived from Type II "S"	4	70 mgm.	10^{-3}	—	1			
			10^{-4}	—	1			
			10^{-5}	—	1	3	none	negative
			10^{-6}	1	—	1	10^{-6}	negative
Supernatant after removal of nucleoprotein	4	30 mgm.	10^{-3}	—	1			
			10^{-4}	—	1			
			10^{-5}	—	—	3	none	negative
			10^{-6}	1	—	1	10^{-6}	negative

mination, being 70 mg. Three weeks following the last immunizing injection, they were given intravenously varying quantities of a virulent Type I Pneumococcus culture. The results, as shown in Table IV, indicate that the immunity induced in these animals was extremely slight; indeed it may be doubted whether any increased resistance was demonstrated. In the sera of none of these animals were type-specific antibodies demonstrable and only one of the sera possessed any protective power for mice, in this case of minimal degree.

The rabbits receiving the second material were given repeated injections, the total amount of solution containing 30 mgm. of protein as estimated on the basis of nitrogen determinations. Active immunity was determined by observing the

results of the subsequent injection of different amounts of Type I Pneumococcus culture. The data are given in Table IV and it is seen that little immunity follows the intracutaneous injection of this material. The sera of none of the rabbits possessed type-specific antibodies and the serum of only one of them protected mice against a minimal infective dose. The sera of these rabbits, however, did contain species-specific antibodies, as was shown by their power to precipitate the "nucleo-protein" and to agglutinate R cells.

The results show, then, that repeated injections of soluble derivatives of Pneumococcus into the skin do not render rabbits resistant to infection. Tillett (5) has already pointed out that rabbits do not acquire an active immunity following intravenous immunization with solutions of Pneumococcus.

TABLE V

Protective Property of Sera of Rabbits Immunized Intravenously after Previous Intracutaneous Immunization

Number of rabbits	Previous immunization	Later immunization	Protection titre for mice	
			Number of sera	Degree of protection
				cc.
6	Intracutaneous Type I	Intravenous Type I	3	0.2
			3	0.1
6	Intracutaneous Type III	Intravenous Type I	3	0.2
			2	0.1
			1	0.01

Protective Properties of Sera of Rabbits Immunized Intravenously Following a Previous Intracutaneous Immunization

It was shown in the preceding communication (1) that previous intracutaneous immunization does not render rabbits incapable of forming type-specific antibodies when they are subsequently given intravenous injections. It was interesting to determine the protection titre of the sera of rabbits immunized first by intracutaneous inoculations and later by intravenous injections. The data presented in Table V show that 12 rabbits were originally immunized by intracutaneous injections of suspensions of heat-killed pneumococci. Six were immunized to Type I and six to Type III pneumococci. At the end of the immunization, none of the sera conferred protection upon

white mice to infection by pneumococci of the respective types, and none contained type-specific antibodies. After a subsequent immunization by intravenous injections of Type I pneumococci the sera of all 12 rabbits not only showed a high agglutination titre (about 1:160) but all conferred a high grade of passive protection upon mice against infection by Pneumococcus Type I.

DISCUSSION

The results of the preceding communication (1) disclose the fact that immunization by the intracutaneous injection of suspensions of heat-killed pneumococci, whether S or R, incites the formation of species-specific antibodies in all rabbits. In the animals immunized with Type III pneumococci no type-specific antibodies were found, and in the rabbits immunized with Type I pneumococci antibodies appeared in only 12 per cent of the rabbits studied. The present study reveals that following intracutaneous immunization rabbits acquire a marked degree of resistance to intravenous infection by pneumococci, and that this is true whether the pneumococci injected be of the same type as those employed in immunization or of a different type. This active immunity is effective even in the absence of type-specific agglutinins.

In general, the sera of rabbits immunized by intracutaneous injections fail to protect white mice against infection by Pneumococcus. However, when pneumococci of Type I are employed in the immunization, about 20 per cent of the sera studied conferred on white mice some degree of protection against infection.

Tillett was the first to point out (5) that the intravenous immunization with R or S cells of any type of Pneumococcus renders rabbits actively immune to infection with Type III Pneumococcus. In a subsequent communication Tillett showed (3) that although the sera of rabbits intravenously immunized to R or S pneumococci failed to protect white mice against infection by Pneumococcus, nevertheless, these sera did confer protection upon normal rabbits. Later Goodner (6) reported that rabbits recovering from an intracutaneous infection with live virulent cultures of Type I Pneumococcus were subsequently immune to infection with pneumococci of the same type. More recently Bull and McKee (7) found that following immunization of

rabbits by subcutaneous and intravenous injections of pneumococci of Type II or Type III and by similar injections of a strain of pneumococci of Group IV the animals were actively immune, and that this immunity was effective against intransal or intravenous injections of pneumococci of Type I. Species-specific antibodies were present in the sera of all the animals. Type-specific agglutinins were present in the sera of the rabbits immunized against Pneumococcus of Type II and in the serum of the animals immunized against a Pneumococcus belonging in Group IV. In previous papers (8, 9) Bull and McKee had demonstrated that rabbits in which cultures of Type I pneumococci had been instilled into the nose were subsequently actively immune to infection with the homologous organism, and this in the absence of demonstrable type-specific antibodies.

On the other hand, the intracutaneous injections of soluble derivatives of pneumococci in rabbits is not followed by an active resistance to infection with these organisms and the sera of these animals fail to protect white mice against infection.

The observations made in this study indicate, then, that an active and broad resistance to infection by Pneumococcus may be present in the absence of circulating type-specific antibodies. Active immunity to pneumococcal infection is usually considered to be associated with the development of demonstrable antibodies which in one way or another react specifically with the bacteria themselves. The sera of animals immunized in the usual manner by repeated intravenous injections usually afford passive protection against infection by corresponding types of Pneumococcus to animals of various species and the passive protection is considered to depend upon the transfer of type-specific antibodies. In rabbits immunized by intracutaneous injections, however, the active resistance to infection which develops has been found to be unrelated to type-specificity as is evidenced both by the absence of type-specific antibodies in the serum and by the fact that the resistance to infection which these animals exhibit is effective, not only against pneumococci of the homologous type, but also against infection with pneumococci of heterologous type. Moreover, the sera of these animals do not, as a rule, confer passive protection upon white mice against infection even by homologous types of pneumococci. It is possible that in the one case the resistance is dependent upon the development of a new property, an acquired *active immunity*, and that

in the other case the resistance depends upon an exaltation of a naturally occurring property, an increase in natural *resistance to infection*, but this cannot be determined from the data at hand.

SUMMARY AND CONCLUSIONS

1. Injection of suspensions of heat-killed pneumococci into the skin of rabbits is followed by an active immunity which is effective against intravenous infection by homologous and heterologous types of Pneumococcus.

2. This form of active immunity may be induced by the injection of S or R strains of Pneumococcus.

3. Intracutaneous immunization with soluble derivatives of Pneumococcus does not induce active immunity to infection.

4. The sera of seventy-nine per cent of the rabbits immunized to Type I Pneumococcus by intracutaneous injections afforded no protection to mice against infection with pneumococci.

5. None of the sera of rabbits intracutaneously immunized to the type-specific Type III (S) pneumococci, to R cells, or to soluble derivatives of Pneumococcus protected white mice against infection.

6. The sera of rabbits immunized first intracutaneously and subsequently intravenously possess a high titre of protective antibodies.

7. It may be concluded that when type-specific pneumococci are injected into the skin they lose the property of stimulating an active immunity of a specific type and of stimulating the production of type-specific antibodies, but they act just as do the degraded or R forms, causing the animals to become resistant to infection with pneumococci of all types without the development of any type-specific antibodies in the serum.

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REACTIONS OF RABBITS TO INTRACUTANEOUS INJECTIONS OF PNEUMOCOCCI AND THEIR PRODUCTS

III. Reactions at the Site of Injection

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It has been shown in a preceding paper (1) that the repeated intracutaneous injection of heat-killed pneumococci in rabbits stimulates the formation of species-specific, rather than type-specific, antibodies.

When Type I Pneumococcus was used as antigen, a small number (12 per cent) of the animals did develop a low serum titre of type-specific antibodies, but in all other instances, type-specific antibodies failed to appear in the serum. In a general way, moreover, the antibody response was the same when either S or R cells, or even soluble derivatives of Pneumococcus, were administered intracutaneously. Following the injections of S or R organisms, rabbits acquired a marked degree of resistance (2) which was effective against intravenous infection by any type of Pneumococcus. The injection of soluble derivatives, on the other hand, induced the development of no active immunity.

When rabbits received an intracutaneous injection of a suspension of heat-killed pneumococci a reaction always occurred at the site of injection, and as the inoculations were repeated week after week with a fixed quantity of bacteria, the local reactions following the injections manifested a change in size, character, intensity, and duration. The occurrence of reactions was in no way related to the type or variety of Pneumococcus employed in the injections nor was the intensity of the reaction dependent upon the culture employed. A study has been made of these local reactions.

EXPERIMENTAL

Inoculations of 0.2 cc. of suspensions of heat-killed pneumococci[1] were made into the skin over the flanks. Repeated injections were made alternately on the two

[1] In all cases, unless otherwise stated, the suspensions of heat-killed pneumococci were prepared in such manner that 1 cc. of the suspension contained the bacteria from 10 cc. of an 18 hour broth culture.

sides and a new location was chosen each time. Descriptions and measurements of the local reactions were made each day until a complete and final disappearance had taken place. The size of the area of skin involved in the reaction, as determined by actual measurement, has been held to be the only available method of quantitative estimation of the intensity of a given reaction. Usually the reactions reach their greatest size about 48 hours after the injection. Consequently, the sum of the two largest diameters, as measured at this time, has been considered to be an index of the intensity of any given reaction. This has permitted a comparison of the reactions occurring after the first and those after the subsequent injections.

Skin Reactions Following Initial Injection

The intracutaneous injection into a rabbit of 0.2 cc. of a suspension of heat-killed pneumococci is followed within a few hours by the appearance at the site of injection of a circumscribed, slightly raised and indurated nodule, measuring from 1 cm. to 1.5 cm. in diameter. The skin over this area is reddish in color. The reaction reaches its maximum size, of from 1 cm. to 1.5 cm. in diameter, in about 36 to 48 hours. It gradually becomes smaller and disappears within 4 to 5 days without the skin breaking down or becoming necrotic. There is some variation in the intensity of the reaction in individual animals but usually the course is about as stated above. Occasionally, regression occurs more rapidly, within two or three days, or, more rarely, complete disappearance is delayed, even for as long as two weeks or more.

Skin Reactions Following Repeated Injections

When after an interval of a week, a second injection is made into the skin the reaction which follows differs somewhat in intensity and character from that observed following the first injection. The modification of the reaction becomes more marked as the injections are repeated from week to week. Reactions of the greatest intensity occur usually after the fourth to the sixth injection.

In the more intense reactions the area of the skin involved reaches 4 cm. to 6 cm. in diameter. The skin is markedly elevated and of a deep red or purplish hue, and at times appears mottled with small yellow areas. Surrounding the raised area there is usually an areola of erythema varying from 0.2 cm. to 1.5 cm. in breadth. Outside of this the skin may be slightly edematous over a considerable area,

especially on the ventral margin. Not infrequently necrosis of the skin occurs, with discharge of purulent material. In case necrosis does not occur, the disappearance of the lesion is delayed. The time required for regression is apparently related directly to the intensity of the reaction.

To illustrate the change which gradually occurs in the intensity and character of the reactions following repeated injections, charts have been made which give the size of the area involved in each reaction, the length of time required for its disappearance, etc.

TABLE I

The Duration of Successive Reactions Following Repeated Intracutaneous Injections of 0.2 Cc. Suspensions of Pneumococcus

Rabbit No.	Number of the injection									
	1	2	3	4	5	6	7	8	9	10
12-56	9*	15	24	23	33	14	12			
12-57	17	16	24	22	37	15	21			
12-58	16	12	22	16	21	7	6			
12-59	22	20	59	28	44	10	10			
12-60	20	17	50	58	37	16	12			
13-89	7	62	55	50	40	31	21			
13-90	10	22	22	21	17	9	16			
13-69	5	21	25	36	32	22	13	9	5	5
13-70	4	18	34	36	31	12	5			
13-72	5	14	43	35	29					

* The figures represent the number of days required for the disappearance of the reactions.

After reactions of maximum intensity occur, following the fourth or fifth injection, the reactions to subsequent injections vary considerably as regards size. At first, there occurs a gradual decrease but later marked variations occur. These variations are partly explained by the fact that the reactions to later injections are frequently less well circumscribed, and, therefore, less easily measured than are the reactions following the earlier injections. This variability in size is shown in Figures 1, 2, and 3.

In Table I is recorded the number of days required for complete disappearance of each reaction which follows each of ten consecutive

injections made at intervals of a week. It will be seen that the number of days required for the lesions to disappear becomes progressively greater up to about the fourth injection, when the duration of the lesion again becomes diminished. This is also shown in the curve in Figure 1. Any marked irregularities which occured were usually due to necrosis of the lesions and discharge of pus, with consequent more rapid resolution. Although reactions to later injections became less intense, of smaller size and of less duration, they never completely fail to appear, even though many injections are made. The above observations indicate that the reactivity of the skin to injections of pneumococci becomes gradually increased at least, up to the time four or five weekly injections have been made.

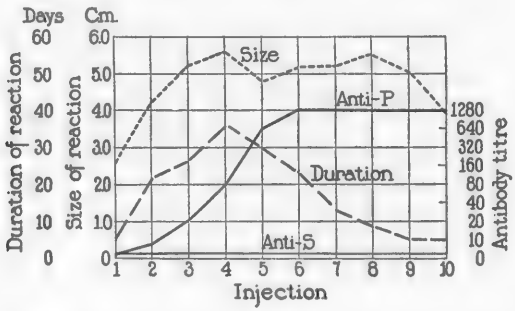

FIGURE 1. The size and duration of the skin reactions following each of a series of injections of heat-killed pneumococci, and the development of antibodies in the serum.

It seemed, however, important to attempt to demonstrate this in another manner, namely, by determining the minimum amount of bacterial suspension required to produce a visible reaction after the animal had received three previous injections, as contrasted with the minimal amount required to produce a reaction in the animal previously untreated.

Each of eight animals received intracutaneous injections of suspensions of heated pneumococci representing amounts of culture varying from 2 cc. to 0.0001 cc. In all cases the volume of fluid injected was 0.2 cc. It was found that reactions occurred when suspensions containing the bacteria from 1 cc. to 0.1 cc. of culture had been injected, but not when suspensions containing smaller numbers

of bacteria were employed. Each of the eight rabbits now received at the end of each of the two subsequent weeks the usual injections of 0.2 cc. of suspensions and the usual reactions were observed. At the end of the following week each of the rabbits received injections of varying amounts of suspension corresponding to those employed in the first test. It was now found that two of the animals showed reactions where amounts of suspension containing the bacteria from .001 cc. of culture were injected, one showed a reaction with an amount as small as that representing .01 cc. of culture, and five showed reactions with amounts of suspensions representing 0.1 cc. of culture. These results are shown in Table II.

After these animals had received injections for three weeks they reacted to amounts of culture smaller than those which were neces-

TABLE II

Minimal Doses of Suspensions of Pneumococcus Which Elicit Skin Reactions in Normal Animals and in Animals Previously Injected

Rabbit No.	Minimal dose eliciting reaction at	
	First injection	Fourth injection
	cc.	*cc.*
8-47	0.01*	0.01
8-48	0.01	0.0001
8-49	0.2	0.01
8-50	0.01	0.0001
8-51	0.1	0.001
8-52	0.01	0.01
8-53	0.1	0.01
8-54	0.1	0.01

* These quantities of bacterial suspension represent a tenfold concentration of culture.

sary to elicit a reaction originally. The previous observations of an increase of reactivity following successive injections were therefore confirmed by these tests.

Relation of the Increasing Skin Reactivity to the Development of Circulating Antibodies

An attempt was made to correlate if possible the increasing skin reactivity described above with the appearance of circulating antibodies. As has already been pointed out in previous communications (1, 2) the appearance of type-specific and protective antibodies following intracutaneous injections of suspensions of heat-killed pneu-

mococci occurs only occasionally and then only when pneumococci of Type I have been employed in the injections. On the other hand, the present study shows that all rabbits develop an exalted skin reactivity following successive intracutaneous injections of heat-killed pneumococci, no matter what type of Pneumococcus is employed. Consequently, skin reactivity cannot be related directly to the appearance of type-specific antibodies in the blood serum.

On the other hand, as has previously been shown, following repeated intracutaneous injections of heat-killed pneumococci all rabbits develop in their serum a high titre of species-specific antibodies. A study of increased skin reactivity and the presence of species-specific anti-

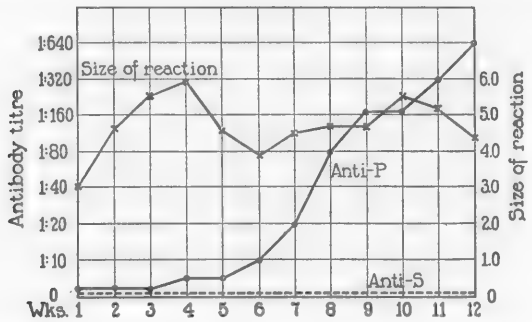

FIGURE 2. The development of antibodies and the size of skin reactions following each of a series of injections of Pneumococcus, Type I.

bodies in the serum was made in a large number of animals, but for the sake of brevity, the observations made on 8 rabbits only will be given in detail as typical. Four rabbits received intracutaneous injections of suspensions of heat-killed Pneumococcus Type I, and four received similar injections of suspensions of Pneumococcus Type III. The size of the reaction at the site of each of the injections was recorded, and the type-specific and species-specific titre of the blood was determined in each animal each week. In Figure 2 (Type I Pneumococcus) and Figure 3 (Type III Pneumococcus) composite curves indicating the increase of skin reactivity and the development of each kind of antibody during the period of the experiment are given. In each instance, it is seen that during the early injections, the skin reactivity

increased rapidly, while species-specific antibodies appeared in the blood only slowly. With the later injections and the approach of the height of the skin reactivity, the content of the serum in species-specific antibodies increased; later although the skin reactivity remained constant or decreased, nevertheless, the species-specific antibodies increased rapidly. It has not been possible, therefore, to demonstrate any direct relationship between the appearance of species-specific antibodies in the blood and the development of heightened skin reactivity.

Previous experiments have indicated that the degree of active resistance which develops following intracutaneous injections also increases

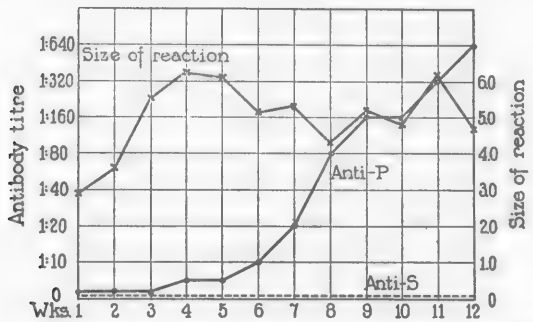

FIGURE 3. The development of antibodies and the size of skin reactions following each of a series of injections of Pneumococcus, Type III.

with the number of injections. It seems possible, therefore, that although a relationship may exist between these two phenomena, the exact nature of this relationship is, at the present time, obscure.

Passive Transfer of Increased Skin Reactivity

Serum obtained from rabbits at the height of skin reactivity was injected intravenously into normal rabbits in quantities varying from 10 to 30 cc. Other normal rabbits were given serum from rabbits which had received repeated intracutaneous injections of egg albumin or Pneumococcus "nucleoprotein." Twenty to twenty-four hours following the transfers, injections of suspensions of heat-killed pneumococcus were made into the skin of all the animals which had re-

ceived the serum. Control normal rabbits which had received no serum were also given similar intracutaneous injections. It was found that the rabbits which had received serum from highly reactive animals, those which had received serum from the animals immunized to proteins, and the normal rabbits all exhibited skin reactions of the same character and the same degree of intensity. It seems, therefore, that the property of increased skin reactivity cannot be transferred to the normal rabbits by intravenous injection of serum from the highly reactive animals.

The Secondary Reaction

Andrewes, Derick, and Swift (3) have shown that when rabbits are injected in the skin with live or dead bacteria, particularly with certain strains of Streptococcus viridans, a local reaction occurs. This begins to retrogress in about 48 hours, but in over half of the animals, about the eighth or ninth day, a recrudescence of the reaction takes place. Among other observations, they showed that when living R pneumococci, derived from a Type I strain, were injected, secondary reactions occurred in 2 of 13 rabbits tested. When the same organisms killed by heat were injected, or when heated S pneumococci of Type I were injected, a secondary reaction occurred in 2 of 15 rabbits.

In the present study secondary reactions have been observed in 31 out of 54 rabbits which received intracutaneous injections of suspensions of heat-killed pneumococci.

The secondary reaction occurred only after the first injections, never following subsequent ones. The secondary reaction after injections of pneumococci usually appears 7 to 12 days after the injection, and usually after the primary reaction has entirely disappeared. The secondary reaction persists from a minimum of 3 days to a maximum of 25 days, the average duration being 12 days. In appearance it usually resembles the primary reaction, being circumscribed, slightly elevated, pink to reddish in color, with little or no edema, and the lesion has never been observed to break down. Secondary reactions have not been observed following the intracutaneous injections of soluble derivatives of pneumococcus cells.

No relationship could be established between the appearance of secondary reactions and the appearance of type-specific or species-specific antibodies in the blood, nor was there any apparent relationship between the appearance of secondary reactions and the development of active immunity.

In the rabbits mentioned in the previous experiment which had received serum from animals highly skin reactive to pneumococci or from animals which had previously received injections of solutions of egg albumin or "nucleoprotein," secondary reactions following the intracutaneous administration of pneumococci were observed in the same frequency as those occurring in untreated animals.

DISCUSSION

The repeated injections of suspensions of heat-killed pneumococci into the skin of rabbits stimulate the development of an increased reactivity of the skin as is shown by the greater size and intensity of the reactions to subsequent injections, and by the fact that doses of heat-killed pneumococci, which in the normal animal are too small to produce any reaction, in the animals which have been previously injected produce marked reactions. The height of increased skin reactivity is reached when 4 to 6 intracutaneous injections have been made, after which the reactivity diminishes. This increased skin reactivity develops after the intracutaneous injection of pneumococci of any type or form, and the reactions are similar, no matter what type or form of Pneumococcus has been employed. There is apparently no relationship between the development of an increased skin reactivity and the appearance of type-specific antibodies in the blood, nor does the development of heightened skin reactivity seem to bear any definite relationship to the appearance of species-specific antibodies. While increased skin reactivity appears at a time when increased resistance to infection is present, the relationship between these two phenomena is still obscure.

The heightened skin reactivity is probably dependent upon some alteration in the tissues themselves, since transfers of the blood serum of highly reactive animals to normal rabbits does not endow the latter with the property of reacting with increased intensity.

Finally, it has been found that a secondary reaction may occur following the primary reaction. This is similar to the reactions following the intracutaneous injections of dead or living cells of Streptococcus viridans as studied by Swift and his associates (3, 4, 5). With pneumococci this secondary reaction only occurs following the first injection, never following the subsequent ones.

SUMMARY AND CONCLUSIONS

1. Following repeated intracutaneous injections of heat-killed pneumococci rabbits acquire an increased skin reactivity.

2. The increased skin reactivity reaches a maximum after 4 to 6 injections have been made, after which it becomes greatly diminished.

3. The relationship of increased skin reactivity to active resistance to infection by Pneumococcus, and to the presence of species-specific antibodies in the blood, is still obscure.

4. The increased skin reactivity is not transferable by serum from a highly reactive to a normal rabbit.

5. After regression of the reaction to the first injection of Pneumococcus into the skin, there frequently follows a recrudescence, or exacerbation, of the reaction.

6. The increased skin reactivity and secondary reactions are incited alike by all types and all forms of Pneumococcus.

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THE IMMUNOLOGICAL SIGNIFICANCE OF COLOSTRUM

I. The Relation between Colostrum, Serum, and the Milk of Cows Normal and Immunized towards B. coli

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The passing on from one generation to another of endemic infectious agents and parasites takes place most frequently at birth or soon after. In some instances the egg is the carrier or intrauterine transmission occurs. The mortality of the young generation is governed largely by the variety, concentration and virulence of the infectious agents and parasites domiciled in the herd or flock. The intrauterine transmission of antibodies towards certain enzootic diseases developed in the female parent and carried in the blood occurs in certain species. In others, such as the bovine species, the temporary protection of the young is accomplished by the storage of antibodies in the udder, quiescent before parturition, and the passage of these through the mucosa of the digestive tract when colostrum is taken during the first, and perhaps the second, day of life. The protection afforded by colostrum is probably limited to certain common, ubiquitous species of bacteria living in the digestive tract and on the mucosa of the respiratory tract. The more formidable invaders, such as tubercle bacilli, may be held off temporarily but no complete immunity is afforded, such as that against pathogenic *B. coli* races. In fact certain pathogenic bacteria tend to parasitize the udder. To prevent the newborn calf's ingesting such infectious agents, efforts have been made to pasteurize colostrum or to replace it with milk, frequently with disastrous results. The importance of colostrum in controlling the early mortality due to *B. coli* has been brought forward in earlier publications.[1] At the same time the possibility of substituting normal cow serum for colostrum

[1] Smith, T., and Little, R. B., *J. Exp. Med.*, 1922, **36**, 181.

was indicated in certain experiments on calves.[2] The use of serum was based on the assumption that it contains at least as much antibody as the colostrum. The experiments to be reported do not wholly substantiate this assumption. In fact the evidence to be presented indicates a much higher concentration in the colostrum than in the serum.

The antibody content towards *B. coli* was singled out, partly because the early diseases of calves are due to members of this group, partly because the antibodies are quantitatively measurable in guinea pigs.

A single strain of *B. coli*, already described,[3] was used in all experiments. This readily mutating strain was employed in the original or (a) form. Several cows were treated subcutaneously and intravenously with living and heated culture suspensions for variable periods of time to furnish a protective serum for control studies.[4] The quantitative estimation of protective antibodies towards *B. coli* was made on guinea pigs weighing 350–400 grams. Exactly 24 hour bouillon cultures were used. The bouillon from the same lot of veal was stored in full bottles in a refrigerator and only this was employed. The minimum fatal dose was determined and 1¼ to 1½ times this dose was mixed with the serum or colostrum to be tested and injected after 15 minutes into the peritoneal cavity. Control tests of virulence of the culture were associated with each separate experiment.

The Relative Content of Protective Bodies towards B. coli in Colostrum and in Serum of Untreated Cows.—The protective action of colostrum having been demonstrated indirectly by withholding this fluid in feeding newborn calves, it seemed desirable to use the test on guinea pigs, described above, to determine quantitatively its protective capacity against the species of bacteria chiefly responsible for the early deaths of calves. The results are given in Table I. To simplify the table and still furnish the information at hand, the amounts of colostrum actually tested are tabulated with the sign (+) indicating protection against the fatal dose, and (—) indicating death of the guinea pig. Lower or higher amounts than those given were not tested. Hence the figures do not in every case represent upper and lower limits. The same strain of *B. coli* (1192a)[3] was used unless otherwise indicated.

Attention is called to certain data in this table. (1) The colostrum of normal cows as a rule contains antibodies to a *B. coli* of the scours

[2] Smith, T., and Little, R. B., *J. Exp. Med.*, 1922, **36**, 453.
[3] Smith, T., and Bryant, Gladys, *J. Exp. Med.*, 1927, **46**, 133.
[4] Smith, T., and Little, R. B., *J. Exp. Med.*, 1930, **51**, 483.

TABLE I

The Antibody Content of Colostrum, Milk, and Serum of Normal Cows towards B. coli

No. of cow	Breed	Source	Entry into herd	Date of test	Dose of			Remarks
					Colostrum (+) = protection	Milk	Serum	
				1926				
1	Holstein	Wisconsin	Aug., 1926	Oct.	0.2 (+)	—	—	
2	"	Native	—	Nov. 3	0.2 (+); 0.4 (+)	0.4 (−)	0.2 (−); 0.4 (−)	Milk drawn 7 days later
3	"	"	—	" 3	0.2 (+); 0.4 (+)	—	0.2 (−); 0.4 (−)	Same result with same colostrum refrigerated 7 days
4	Jersey	Oregon	June, 1926	" 15	0.2 (−); 0.4 (−)	—	—	
5	"	"	" 1926	" 19	0.2 (−); 0.4 (+); 0.6 (+)	—	—	
6	Guernsey	Michigan	Oct., 1926	" 19	0.2 (−); 0.4 (+)	—	—	
7	"	Wisconsin	April, 1925	" 24	0.2 (+); 0.4 (+); 0.6 (+)	—	—	
8	Holstein	Native	—	Dec. 3	0.1 (+); 0.075 (−); 0.05 (−)	—		
9	"	"	—	" 7	0.1 (+); 0.075 (−); 0.05 (−)	—	—	
10	"	Michigan	Sept., 1922	" 11	0.1 (+); 0.05 (−)	—	—	
				1927				
11	Jersey	New Jersey	Feb. 2, 1927	Mar. 10	0.2 (+)	—	—	
12	Holstein	Native	—	" 10	0.1 (−); 0.2 (+)	—	—	
13	"	Michigan	Feb. 27, 1927	Apr. 7	0.2 (−); 0.4 (−)	—	—	Colostrum thin, due to loss on account of overdistension of udder and leakage
14	Guernsey	Native	—	" 13	0.2 (−); 0.4 (−); 0.6 (+)	—	—	

type. 0.1 cc. to 0.2 cc. may protect guinea pigs against the surely fatal dose. Tests with quantities less than 0.1 cc., when made, were negative. The protective capacity of certain samples was low. Thus No. 4 failed in 0.4 cc. doses, Nos. 5 and 6 in 0.2 cc. doses. To interpret this deficiency, the dates of entry into the herd are given. These cows came in a short time before calving. The inference, based on perhaps too few cases, is that they were not yet immunized to the new flora. On the other hand, No. 1 came within the range of the native cows and the native No. 14 was low. (2) The milk following the colostrum has lost more or less of its protective power, as shown by No. 2. (3) The

TABLE II

The Relative (Protective) Antibody Content of Serum and of Milk of Two Treated Cows and a Normal Cow

No. of cow*	Treatment	Serum titer	Milk titer	Agglutination titer of serum towards 1192_b (mutant)	$\frac{\text{Milk}}{\text{Serum}}$ (protection)
D	Normal	1.0 (−)	0.8 (−)	—	—
A	Living cultures	0.005 (+)	0.6 (+); 0.4 (−)	1:1,280	$\frac{1}{120}$
B	Heated "	0.005 (+)	0.6 (+); 0.4 (+); 0.2 (+); 0.07 (−)	1:2,560	$\frac{1}{40}$

* For data on the treated cows see *J. Exp. Med.*, 1930, **51**, 483.

serum of normal cows drawn at the same time that the colostrum was obtained is distinctly below the colostrum in antibody content. Thus the sera of Nos. 2 and 3 failed to protect in 0.4 cc. doses. The colostra protected in 0.2 cc. doses and probably in less. Earlier, fairly numerous tests of normal cow sera failed to show any protection in doses of 1 and even 2 cc. Hence tests of normal sera were omitted with exceptions noted.

The Relative Antibody Content of Serum and Milk of Immunized Cows.—A number of tests were made to determine the protective titer of the milk of immunized cows. In both cows treated with living and heated cultures, respectively, the milk titer rose in the first to 0.6 cc.,

and in the second to 0.2 cc. Owing to the fact that the original type of *B. coli* used failed to become agglutinated in the immune sera except in very low dilutions, the mutant was used in agglutination tests. Since the immunized cows were not bred, no colostrum from them was available.

The Effect of Later Feedings of Immune Serum on the Antibody Content of the Blood.—The significance of lactation on the transfer of immune bodies after the two first days of life has not been satisfactorily cleared up in the earlier experiments. The extensive literature on this subject is more or less contradictory, probably due to the use of different species of animals and alien serum. In a former article[5] the absorption of antibodies in the form of *B. abortus* agglutinins from the digestive tract of calves was prompt and abundant when fed in immune cow serum during the early hours of life. One calf fed a serum of high titer when $3\frac{1}{4}$ days old showed no increase of agglutinins thereafter. It was furthermore shown that the feeding of milk of high titer was less favorable to the absorption than either colostrum or serum.

The two following cases are further contributions to this subject. Homologous serum was fed to calves $2\frac{1}{4}$ and 18 days old, respectively. In neither animal was an increase in agglutinins demonstrated as a result of the feeding.

Calf 1430. Holstein bull calf, weighing 50–55 lbs., born April 1, 5.30 a.m. Weak and unable to stand at first. Held up to the udder for the first meal when 3 hours old. Left with dam until April 3, 2 a.m. At 10.50 a.m., when about $2\frac{1}{4}$ days old, it was fed with immune serum of Cow B (see Table II) mixed with milk from the same animal. It drank about 350 cc. of serum. At 7.40 p.m. the calf drank about 250 cc. of serum diluted with milk. Animal thereafter fed milk drawn from the udder of the dam.

April 26. Calf has been normal throughout excepting for a short period of soft, whitish fecal discharges a week ago. The agglutinin titer of the calf's serum before and after the feeding of the immune serum is given in Table III. For comparison a sample of the dam's serum is included.

It will be noted that the dam's colostrum had already given the calf's serum a considerable amount of agglutinins towards *B. coli* 1192_b. The feeding of immune serum later failed to raise the titer. The serum titer of the dam was only a little higher than that of the calf.

[5] Smith, T., and Little, R. B., *J. Exp. Med.*, 1923, **37**, 671.

The explanation of this slight difference may be looked for in the high concentration of *B. coli* antibody in the colostrum.

In view of the relation existing between the protein output of the urine and the intake of colostrum during the first days of life[6] the protein content of the urine was taken into consideration. When the calf was 29 hours old and before the serum feeding the protein in the urine was sufficient, when the heat test was applied, to produce a coagulum deposit after 24 hours sedimentation equivalent to 15.3 per cent of the volume of the entire fluid. Fourteen hours after the second

TABLE III

Titrations of Serum of Calf 1430 against B. coli 1192$_b$ after Ingesting 600 Cc. Serum of Titer 1:2,560

Date of collection of serum	Serum dilutions							
	1:10	1:20	1:40	1:80	1:160	1:320	1:640	1:1,280
April 3, 10.50 a.m. (just before feeding serum)	C	C	C	++++	+++	+	−	−
April 3, 7.40 p.m. (just before 2nd feeding of serum)	C	C	C	++++	+++	++	±	−
April 4, 9.05 a.m.	++++	++++	++++	+++	++	+	−	−
Titration of Dam's Serum								
April 4, 9.10 a.m.	C	C	C	C	+++	++	−	−

feeding of serum, when the calf was 3 days and 4 hours old, the deposit was only 5.3 per cent, and on the following day it was 1.3 per cent. These figures do not present any evidence that any excess of protein had been absorbed from the serum fed.

Table IV shows the absence of any protection transmitted by the immune serum to the calf's serum.

Calf 1412. Fed serum and milk of untreated Cow 1229 at start, with only one discharge of fluid feces on 2nd day. When 18 days old, it was fed 1,000 cc. of

[6] Smith, T., and Little, R. B., *J. Exp. Med.*, 1924, **39**, 303.

immune serum of Cow A (see Table II) administered in 3 doses of 500, 300, and 200 cc. respectively, in one day. On the following day blood was withdrawn for tests 24 and 30 hours after the first feeding of serum, and 12 and 18 hours after the third or last feeding. Neither sample showed any appreciable difference in its agglutinin content from the sample drawn 5 days before the feeding. The urine of

TABLE IV

Protective Titer of Serum of Calf 1430 Before and After Ingestion of Immune Serum

Guinea pig No.	Dose of *B. coli* culture	Dose of serum	Result
	cc.	*cc.*	
1	0.04	—	Dead in 9 hours
2	0.044	—	" " 8 "
3	0.044	1.0 (a)*	" " 6 "
4	0.044	0.5 (b)	" " 9 "
5	0.044	1.0 (b)	" " 8 "
6	0.044	0.5 (c)	" " 6 "
7	0.044	1.0 (c)	" " 8 "

* (a) calf serum drawn immediately before feeding; (b) 9 hours after first and just before second feeding; (c) 13 hours after second feeding.

TABLE V

Calf 1416

Agglutinin Titer of Serum before and after Newborn Calf Had Been Fed Serum of Cow B (Heated Cultures) and Normal Milk

Blood drawn	Serum dilutions								
	1:10	1:20	1:40	1:80	1:160	1:320	1:640	1:1,280	1:2,560
Feb. 1, 5.45 a.m.	++	+	+	±	−	−	−	−	−
" 2, 3.10 p.m.	+++	+++	++++	++++	++	+	−	−	−
Serum of Cow B	C	C	C	C	C	C	C	++++	++++

the two following days was as before the feeding. It showed only a very faint clouding after the heat test. Tests on guinea pigs did not indicate any increase in resistance referable to the serum.

Contrasting with these cases is that of another calf (No. 1416, Table V) fed immune serum in place of colostrum during the 1st day of life.

The agglutinins towards *B. coli* (*b*) were tested in the calf's serum before and after the feeding of immune serum. Serum drawn before the first meal produced a slight deposit of clumps in 1:10 dilution. Twenty-one hours after the first meal the clumping was nearly complete at 1:80. There was a trace at 1:320. The immune serum itself showed nearly complete clumping at 1:2,560. Blood drawn 26 days later had declined in agglutinin content from 1:160 to 1:20.

The protective power of the same samples of serum was distinctly increased by the feeding. The sample drawn before the first meal failed to save the life of the guinea pigs in 1 cc. doses, whereas the sample following the feeding protected in 0.5 cc. but not in 0.25 cc. doses. The serum drawn 26 days later did not protect in a 0.75 cc. dose.

TABLE VI

Calf 1416

Protective Power of Serum before and after Calf Had Been Fed Serum of Cow B

Dose of *B. coli*	Dose of serum	Result
cc.	*cc.*	
0.036	—	Dead in 6 hours
0.04	—	" " 8–9 "
0.04	1.0 (a)*	" " 8–9 "
0.04	0.5 (b)	Lives (40 gram loss in 3 days)
0.04	1.0 "	" (30 " " " 3 ")
0.04	1.0 (a)	Dead in 6 hours
0.04	0.25 (b)	" " 8–9 "
0.04	0.5 "	Lives (no loss in 2 days)

* (a) = serum before feeding; (b) after feeding.

In another calf (1431)[7] fed serum on the 1st day the agglutinin titer rose from /20 to /160. Eleven days later the titer had receded considerably. One cc. of the serum drawn before the first meal failed to save the life of a guinea pig. Serum drawn 23 hours later protected in 1 cc. but not in 0.5 cc. doses. In still another calf (1417) treated in the same way the agglutinin titer rose from /20 to /80 and the protective dose from above 1 cc. to 0.5 cc. On the other hand, in a third calf (1437) neither agglutinin nor protective antibody increase was detected. The calf, however, continued normal until killed when 1¾ months old.

Another method of determining the end period of effective colostrum feeding is to postpone it. Here the danger of early invasion by in-

[7] These figures refer to calves in the second paper of this series, *J. Exp. Med.*, 1930, **51**, 486.

fectious agents must be taken into consideration. In two calves reported upon elsewhere[8] the feeding of colostrum delayed 12 and 18 hours, respectively, was not followed by abnormal conditions during the 2 months' life of the calves.

SUMMARY

The protective antibody content of normal cow serum is below that of colostrum of the same animal. The method used does not permit the titration of the actual amount of the antibody in serum. Quantities up to 2 cc. have no protective effect. The same limitations apply to the titration of milk owing to the introduction of large quantitites of foreign protein into the peritoneal cavity of the guinea pig. When cows were immunized and a serum of high titer obtained, the antibodies in the milk of such cows rose to within the range of the method of testing. The relation of the protective capacity of serum to that of milk was approximately $\frac{1}{120}$ and $\frac{1}{40}$ in the two animals. These figures do not differ much from those obtained by early investigators titrating the antitoxic content of serum and milk of animals undergoing immunization with diphtheria toxin. In the two experiments on calves, $2\frac{1}{4}$ and 18 days old respectively, fed a highly protective serum, no increase in agglutinins or protective antibodies could be demonstrated. The postponement of colostrum to the 12th and 18th hour, respectively did not prevent normal growth.

[8] Smith, T., and Little, R. B., *J. Exp. Med.*, 1930, **51**, 488.

THE IMMUNOLOGICAL SIGNIFICANCE OF COLOSTRUM

II. The Initial Feeding of Serum from Normal Cows and Cows Immunized towards B. coli in Place of Colostrum

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It has been shown that in place of colostrum normal cow serum fed and injected will protect most calves so treated against the various early diseases associated with *B. coli*. It has also been shown that in normal cow serum the specific protective bodies against *B. coli* are much weaker than those in colostrum and below the range of values detected by the intraperitoneal injection of mixtures of cultures and serum into guinea pigs.[1] It may be assumed that colostrum, and cow serum in much less degree, contain antibodies towards other endemic infectious agents which are held in more or less complete subjection in older animals. Hence the feeding of a serum containing a high concentration of *B. coli* antibodies might not protect to the same degree against other diseases developing later and more slowly. However, it was thought best in carrying out the following experiments to try the serum of cows hyperimmunized towards *B. coli*, to keep the serum monovalent, and to administer it only by mouth in full imitation of the natural intake of colostrum. Calves were also fed with normal cow serum. Methods for testing the relative protective power of the sera against *B. coli* on guinea pigs are given in another paper.[1]

The Immunization of Cows.—To provide the immune serum cows were treated in several different ways. *B. coli* strain 1192_a[2] was chosen. This was a highly virulent, hemolytic race, not fermenting saccharose. The cows received both intravenous and subcutaneous injections variously spaced in time according to the condition of the animal. One cow was treated with living cultures, another with

[1] Smith, T., *J. Exp. Med.*, 1930, **51**, 474.

[2] Smith, T., and Bryant, G., *J. Exp. Med.*, 1927, **46**, 133.

cultures heated at 62°C. for 30 minutes, a third received heated cultures of the mutant designated 1192_b, and a fourth bouillon culture filtrates. All intravenous injections were followed by more or less prompt reactions on the part of the respiratory system similar to the disturbances produced in calves by the intravenous injection of filtrates.[3] To avoid too great depression as a result of intravenous injections, the subcutaneous route was frequently used. Large local swellings appeared with or without a febrile reaction, which always followed intravenous administration. There was evident a tendency towards hypersensitiveness when the treatment had progressed 6 or more months. The doses then had to be reduced accordingly.

Table I gives a few data concerning these treated cows. The protective titer of the sera as tested on guinea pigs was about the same for

TABLE I

Immunization of Cows to B. coli

No. of cow	Breed	Strain of *B. coli*	Treatment	Treatment begun	Treatment terminated	Protective titer of serum
						cc.
1109	Guernsey	1127_a	Living culture	Nov., 1924	April, 1925	0.1
A	Holstein	1192_a	" "	" 1925	July, 1927	0.005
B	Jersey	1192_a	Heated "	Sept., 1926	" 1927	0.005
C	Guernsey	1192_b	" "	Dec., 1927	March, 1928	0.02*
D	"	1192_a	Culture filtrate	Nov., 1926	June, 1927	0.5

* Towards mutant.

the cows treated with living and heated cultures respectively. The 48 hour bouillon filtrates failed to produce a potent serum. The serum of the cow treated with heated cultures of the *B. coli* mutant had little or no protective power over the original or (a) form but was quite effective in presence of the mutant.

The Feeding of Newborn Calves with Serum from Normal and Immunized Cows in Place of Colostrum. The method adopted for handling the calves selected for the serum treatment did not differ appreciably from that used in earlier work.

The calf was taken from the dam immediately after birth to the warmed Department units and thoroughly dried by rubbing with towels. Every effort was made to keep the animal protected from undue exposure.

[3] Smith, T., and Little, R. B., *J. Exp. Med.*, 1927, **46**, 123.

The serum was fed from a bottle, provided with a nipple, in three doses, each time mixed with milk. In the first and the second group, the first dose, given as soon as possible and usually within an hour after birth, was about 400 cc. serum. The second dose of 200 cc. was given about 6 to 7 hours later, and the third of 100 cc. from 6 to 10 hours after the second. The total amount of serum, 600 to 700 cc., was thus fed within the first 18 hours of life. In the third group larger amounts were given, also in 3 doses. Thereafter milk from cows well along in lactation was fed 3 times a day, at first from bottles and them from a pail. After 4 weeks some hay and grain were added to the ration.

For agglutination and protection tests blood was withdrawn from a jugular by piercing it with a hollow needle and allowing the blood to run into sterile containers. The tests were made as described in another article.[1]

Urine was obtained whenever possible either when passed voluntarily or by gentle manipulation of the external genitals. The examination was limited to the following operations. The deposits of cloudy fluid thrown down with the centrifuge were examined microscopically. Cloudy samples were filtered until clear. The clear urine was slightly acidified with acetic acid, brought to boiling in a graduated centrifuge tube, and the bulk of any coagulum which had formed measured after 24 hours sedimentation. This quantity divided by the total volume of urine in the graduate is given as per cent protein precipitate. There were also recorded the specific gravity, color, and reaction to litmus. The temperature was taken twice daily. Most of these data have not been reproduced in this paper.

The experiments were carried out on 3 groups of calves in 3 successive years. The gross results are given in Table II, III, and IV. Each group will be briefly discussed and the results summarized.

First Group (1926–7).—Two calves fed milk from an immune cow both succumbed within a few days after birth. The lesions indicated *B. coli* septicemia associated with unrestricted multiplication of *B. coli* in the small intestine. Nine calves which were fed serum of cows treated with living and dead *B. coli* cultures lived beyond the scours period without manifesting any appreciable disturbance. The serum of the fed calves in nearly all cases contained a demonstrable increase of *B. coli* agglutinins and antibacterial substances. Of three calves receiving serum of an untreated cow, one died on the 9th day. One calf receiving serum of the filtrate-treated cow (D) survived, the other died when 4 days old.

Subsequent happenings in calves kept until at least a month old presented new problems. Two manifested symptoms referable to disturbances of the central nervous system. One of these and three others developed a renal disease differing essentially from that described as the "white-spotted kidney" due to *B. coli*.[4] Two were killed too soon (12 and 21 days respectively) to warrant the inference that

[4] Smith, T., *J. Exp. Med.*, 1925, **41**, 413.

TABLE II

*First Group. Calves Fed Serum From Normal and Immunized Cows (600–700 Cc.)**

No.	Breed and sex	Date of birth	Weight at birth	Fed serum of cow No.	Weight at death	Result
		1926	*lbs.*		*lbs.*	
1369	Guernsey male	May 20	55	(Milk only of A**)	—	Dead in 2 days. *B. coli* septicemia.
1396	" "	Nov. 24	—	Normal serum	—	" " 9 " Hemorrhages in 4th stomach.
1401	Holstein "	Dec. 2	90	" "	92	Killed in 12 days. Slightly fatty liver and renal congestion.
1403	" female	" 7	100	A	127	Killed in 21 days. Normal.
1405	" male	" 8	85	A	149	" " 34 " "
1408	" "	" 21	100	A	—	Sold " 23 " "
		1927				
1411	" "	Jan. 2	70	D***	165	Killed in 77 days. Renal lesions.
1412	" female	" 14	70	Normal serum	—	Used in another experiment when 34 days old.
1416	" "	Feb. 1	70	B****	—	Killed when 97 days old. Renal lesions. Blindness.
1417	Guernsey "	" 4	80	B	—	Killed when 97 days old. Normal.
1418	Holstein "	" 9	70	A	94	Dies when 30 days old in convulsions.
1421	Jersey male	March 1	50	D	—	" in 4 days. Hemorrhages in 4th stomach. Scours.
1423	Holstein "	" 3	85	(Milk only of A)	—	Dies in 5 days. Scours. Peritonitis.
1430	" "	April 1	50	(Control)	214†	Killed when 96 days old. Normal.
1431	" "	" 6	85	B	210	" " 75 " " "
1434	Guernsey female	" 13	40	A	56	Moribund when 44 days old. Hemorrhages in 4th stomach. Renal lesions.
1437	" "	May 5	70	A	130	Killed when 2 months old. Normal.
1449	" male	" 27	90	(Control)	137†	" " 1 month " "
1450	Holstein "	" 27	85	"	138†	" " 6 weeks " "

* The 3 first calves received 600 cc., the rest 700 cc.

** Cow treated with living *B. coli*.

*** Cow treated with *B. coli* filtrates.

**** Cow treated with heated *B. coli*.

† 9 days before slaughter.

TABLE III

Second Group. Calves Reared Normally or Serum-Fed

No.	Breed and sex	Date of birth	Approximate weight at birth	Fed serum of cow	Weight at death	Result
		1927	*lbs.*		*lbs.*	
1461	Holstein male	Sept. 6	119 (at 6 days)	(Control)	171	Killed, 1 mo. 17 days old. Normal.
1466	Jersey "	" 21	40	B	134	" 2 " 8 " " "
1467	Guernsey "	" 21	76	(Control)	184	" 2 " 23 " " "
1469	" "	" 28	80	A	114	Dies when 43 days old in convulsions.
1470	Holstein "	" 28	85	B	220	Killed, 2 mo. 15 days old. Normal.
1473	Guernsey female	Oct. 28	75	(Control)	150	" 2 " 8 " " "
1477	" "	Dec. 12	70	A	142	" 1 " 25 " " "
1478	" male	" 12	60	A	—	" 2 " 1 day " "
1479	Holstein "	" 13	85	(Control)	149	" 1 " 17 days " "
1480	" "	" 28	80	B	181	" 1 " 30 " " "
		1928				
1485	Guernsey "	Jan. 10	65	(Control)	141	" 2 " 2 " " "
1492	Jersey "	Feb. 13	55	A	135	" 1 " 27 " " "
1493	Holstein female	" 17	80	A	171	" 1 " 30 " " "
1494	" male	" 14	85	(Control)	208	" 2 " 9 " " "
1498	" "	March 14	85	(Milk only of B)	91	Dies in 37 hours. *B. coli* septicemia.
1499	Guernsey "	" 15	60	A	92	Killed, 2 mo. old. Scours. *B. coli* cystitis.

TABLE IV

Third Group. Calves Reared Normally or Serum-Fed

No.	Breed and sex	Date of birth	Approximate weight at birth	Cow serum fed and amount	Weight at death	Result
		1928	*lbs.*		*lbs.*	
1511	Guernsey male	May 22	—	C, 700 cc.	140	Dies when 1 mo. 28 days old. Renal lesions.
1513	Holstein "	Aug. 16	80	(Control)	158	Killed " 1 " 15 " " Normal.
1514	Guernsey "	Oct. 1	65	C, 700 cc.	133	" " 2 " 2 " " "
1516	" "	" 3	60	C, 700 cc.	112	Sold " 2 " 4 " " Clinically normal.
1518	" "	" 4	55	(Control)	113	" " 2 " 3 " " " "
1522	Holstein "	Nov. 4	85	"	202	Killed " 2 " 1 day " Normal.
1529	" female	Dec. 12	85	Normal serum, 1400 cc.	156	" " 2 " old. Spotted kidney.
		1929				
1531	" male	Jan. 4	110	(Control)	164	" " 2 " 4 days old. Normal.
1532	" female	" 11	75	Normal serum, 700 cc.; D, 700 cc.	147	" " 2 " 8 " " Slight renal lesions.
1533	" male	" 12	95	A, 145 cc.; C, 1260 cc.	172	" " 2 " 3 " " Normal.
1542	" female	Feb. 11	75	Milk only	120	Dies " 1 " 22 " " Large abdominal abscess.
1549	Guernsey male	March 7	75	(Control)	160	Killed " 2 " 16 " " Normal.
1553	Holstein female	" 19	100	Colostrum delayed 18 hr.	190	" " 2 " 1 day " Intestines congested.
1554	" male	" 20	85	Normal serum, 700 cc.	170	" " 2 " 3 days old. Normal.
1556	" "	April 8	85	Colostrum delayed 12 hr.	185	" " 2 " 4 " " "
1562	Jersey female	" 15	45	Normal serum, 700 cc.	93	" " 1 " 27 " " "

they would have remained normal indefinitely. Three of the calves had hemorrhages of the mucosa of the fourth stomach. This condition, to which attention has been called in previous publications, is probably the result of prenatal causes not yet defined. The hemorrhages vary from pale red dots to active petechial, closely set hemorrhages covering the entire mucosa. They may disappear quickly or continue to discharge blood for weeks and are probably the immediate or remote contributory cause of death in such animals. They may have been responsible for the death of Calf 1434. If we exclude this animal, there is one tardy death among 6 fed with serum of Cow A and one calf with renal disease among 3 fed with serum of Cow B.

Three control calves kept with their dams for a few days and then put under the same conditions as the experimental calves remained normal.

Second Group (1927–8).—In planning the experiments of the first group, the possibility that calves which had successfully weathered the early dangerous *B. coli* period might suffer from diseases developing later was not fully realized. Some calves were killed too early. The pathological conditions observed in the first group and in earlier published studies led to various conjectures as to the nature and causes of the conditions. To eliminate some of these a second group was subjected to the same treatment and a few more controls were introduced. These remained with the dam from 4 to 6 days and were then transferred to the Department units. This group was kept under observation long enough to permit the evolution and closer study of the diseases which had appeared in the first group. All serum-fed calves received 700 cc. serum.

From Table III it will be noted that there were 6 controls, 9 serum-fed and 1 milk-fed calf. Those surviving were kept at least 1½ months and most of them 2 months and longer. At the end of this period they were killed and autopsied. All controls remained normal. Of the serum-fed animals, one died in convulsions when 43 days old. One (1499) went through several attacks of scours and later developed a *B. coli* cystitis. The calf receiving milk only from Cow B died of *B. coli* septicemia.

Third Group (1928–9).—In this group were 16 calves. The treatment was the same as in the other groups, excepting that the amount and source of the serum fed differed from those of the earlier groups and from animal to animal. Five calves were introduced as controls. Three were fed with serum of Cow C (mutant *B. coli* treatment), and 3 received normal cow serum. One received normal serum and serum of Cow D (*B. coli* filtrate serum) in equal amounts; another chiefly C serum plus 1/10 A serum. In two the first feedings were with milk and colostrum feeding was delayed 12 hours and 18 hours respectively. One was fed milk only. This animal lived nearly 2 months and died within 15 minutes in convulsions. The autopsy showed the presence of a large abdominal abscess, originating in the track of the urachus. All 5 controls remained normal. Of the serum-fed, one died with renal disease. A second, although with normal

clinical history and autopsy, had slightly heavier kidneys and intranuclear bodies in the uriniferous tubules to be described more fully in another paper.[5]

DISCUSSION

The outcome of the three experiments shows that feeding an adequate amount of serum (600 cc. or more), either from normal cows or from those immunized with one type of *B. coli*, in place of colostrum prevented the early fatalities attributable to *B. coli*, but did not protect all calves from renal lesions or fatal convulsive attacks during the 2nd month of life. That the serum is somehow negatively responsible for these delayed morbid conditions is indicated by the significant fact that all 11 control calves of the second and the third group remained normal whereas of the 17 serum-fed animals of these same groups 2 died. The greater success in preventing disease in these groups as compared with the first group cannot be adequately interpreted in default of knowledge concerning the causes of the convulsive symptoms and renal lesions. The rarity and unexpected appearance of these conditions have interfered thus far with a more thorough study of them.

The age of the serum, normal and immune, varied considerably. Some of it was nearly 2 years old when fed. The guinea pig tests indicated very little antibody decline when the serum was kept refrigerated in nearly full containers. All of the serum was passed through Berkefeld filters and stored without the addition of antiseptics.

The value of the monovalent *B. coli* serum as compared with that of normal cows could have been determined only by a large series of tests on account of the genetically variable animal material, the fluctuating conditions of seasons and of the dairy cow during pregnancy. However, the complete history of the calves, of which the details have been omitted, indicated a greater freedom from early diarrheas among those fed immune than among those receiving normal serum. The experiments bring out the fact that there is nothing qualitatively different in colostrum from normal serum but that the former is quantitatively more effective so far as the content of antibodies is involved. It should be borne in mind that the normally fed calf receives an amount

[5] Smith, T., *J. Exp. Med.*, 1930, **51**, 519.

of colostrum much larger than the serum fed. It would require 2 to 3 liters of normal serum to match the antibody content of the usual amount of colostrum ingested. The inference is thus permitted that there is a large factor of safety in the normal feeding of the newborn calf. That colostrum contains favoring substances not present in serum, as has been claimed at times, is not denied, but the experiments indicate that a sufficient quantity of serum is in 9 out of 10 cases capable of replacing colostrum without injury to the calf. The experiments also indicate that the chief and perhaps the only function of colostrum is to protect the young animal against an early invasion of miscellaneous semi-pathogenic microorganisms by way of the digestive and the umbilical tract.

The pathological conditions appearing in the 2nd month in the serum-fed calves are probably not new diseases but such as are occasionally encountered in calves fed naturally. In the practical application of serum feeding to eliminate contact with the dam immediately after birth, it is suggested that the method might be distinctly reenforced by two or three subcutaneous injections of small quantities of serum during the first 24 hours of life.

The advantages of immune serum do not appear to outweigh the cost of production and it is suggested that the normal serum be used, preferably in larger quantities, such as 1200 to 1500 cc. If possible, the source of the serum should be the same herd or at least herds in the same section of the country, until further experiments should demonstrate no important differences between, let us say, serum from the eastern states and that from the middle or western states. The interchange of cattle is, however, of more importance than locality in the dissemination of infectious agents that tend to become endemic.

The feeding of milk alone, even from highly immunized cows, is again shown powerless to prevent rapid or delayed death.

Though the outcome of these experiments appears on the surface of little practical value, it may be safely predicted that the encroachment of the slowly developing, insidious infectious agents—such as tuberculosis, contagious pleuro-pneumonia, paratuberculosis, and diseases due perhaps to other still undefined agents—will be more dangerous in the dairy herds of the future as they continue to grow in size. In the thoroughbred Guernsey and Jersey cattle the spread of paratubercu-

losis may eventually compel the use of some procedure which separates the calf from its dam at birth.

CONCLUSION

Under certain safeguards, such as isolation, calves from a large dairy herd have been raised by feeding normal and immune cow serum in place of colostrum. The losses were about one out of ten in the later experiments. This outcome may probably be improved by the subcutaneous injection of serum during the first day. This loss may be no greater than that under ordinary conditions, since sporadic deaths among calves are not infrequent. However, no satisfactory statistics are available for comparison with results as given above.

THE RELATIONSHIP OF YELLOW FEVER OF THE WESTERN HEMISPHERE TO THAT OF AFRICA AND TO LEPTOSPIRAL JAUNDICE*

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In the spring of 1928 the accumulated evidence concerning the etiology of yellow fever presented seeming discrepancies which suggested that more than one independent disease had been investigated as yellow fever. To help overcome this confusion the International Health Division of the Rockefeller Foundation undertook to bring together in one laboratory the yellow fever viruses of West Africa and South America and to determine their relationship. The study was begun in June, 1928, at The Rockefeller Institute for Medical Research.

The conflicting evidence referred to may be summarized briefly.

In Cuba in 1900 and 1901 the Yellow Fever Commission of the United States Army under Major Walter Reed (1) found that the etiological agent of yellow fever was present in the circulating blood during the first three days of fever; that it was not cultivable by any of the bacteriological methods used; that it would pass a Berkefeld filter capable of holding back *Staphylococcus pyogenes aureus;* and that it could be readily transferred from a sick person to a well one with the production of infection, by the mosquito *Aëdes aegypti.* The bite of even one mosquito was sufficient to cause the disease.

From 1918 to 1924 a leptospira was isolated by Noguchi and other investigators (2) from patients in outbreaks of yellow fever in Ecuador (Guayaquil), Mexico (Mérida and Vera Cruz), Peru (Morropón), and Brazil (Palmeiras). Like the specific agent studied by the commission under Walter Reed this organism was present in the blood early in the disease, it was not cultivable in the ordinary

* The studies and observations on which this paper is based were conducted with the support and under the auspices of the International Health Division of the Rockefeller Foundation. Laboratory facilities were made available in New York through the courtesy of The Rockefeller Institute for Medical Research.

culture media for bacteria, and it was able to pass Berkefeld filters (V and N). It seemed to differ, however, in not being easily transferable by *Aëdes aegypti*. Noguchi (3) found that he could infect guinea pigs with this leptospira, which he named *Leptospira icteroides*, by means of the bites of *Aëdes aegypti* which had fed previously on a patient or on infected guinea pigs, but that transmission was obtained infrequently considering the number of mosquitoes employed. Another difficulty in the way of accepting the organism as the causative agent of yellow fever was the uncertainty of the diagnosis in the cases in which leptospirae were isolated. In the individual case, infectious jaundice, caused by *Leptospira icterohaemorrhagiae*, is ordinarily indistinguishable from yellow fever on the basis of symptoms. Elliott (4), who was associated with Noguchi in 1918 as clinician of the commission which studied yellow fever in Guayaquil, held the following opinion: "Clinically yellow fever is similar to infectious jaundice. The differences existing between the two diseases appear to be chiefly those of degree. There is more marked jaundice and less hemorrhage in yellow fever than in infectious jaundice." In the differential diagnosis between these diseases the nature of the prevailing epidemic was of necessity frequently allowed to determine the decision with regard to the individual case. To meet this difficulty Noguchi made comparative studies of *L. icteroides* and *L. icterohaemorrhagiae* and decided (2) that they were distinct morphologically and serologically, and that the evidence appeared to warrant the conclusion that *L. icteroides* is the cause of yellow fever. This view became generally accepted.

In 1925 the West African Yellow Fever Commission of the Rockefeller Foundation undertook the study of a disease in West Africa known as yellow fever and indistinguishable from the yellow fever in South America by clinical or pathological characteristics. As summarized by Stokes, Bauer, and Hudson (5), 67 cases of yellow fever were studied bacteriologically by Muller, Kligler, Sawyer, and Bauer between January, 1926, and May, 1927. Although a large number of cultures were made and many guinea pigs inoculated, no leptospira could be isolated nor any other organism which could have a relation to the disease. Yellow fever in West Africa was obviously not due to *L. icteroides*.

In 1927 it was discovered by Stokes, Bauer, and Hudson (5) that the monkey *Macacus rhesus* is susceptible to yellow fever. Using this monkey as an experimental animal they found that the specific agent of the yellow fever of Africa resembled that of the classic yellow fever studied by the army commission in Cuba in being present in the blood early in the disease, in not being cultivable, in filtrability, and in ease of transference by means of *Aëdes aegypti*.

In 1928 Aragão (6) transferred the yellow fever of South America from patients to *Macacus rhesus* monkeys during an epidemic in Rio de Janeiro. The infectious agent was found to be filtrable and was easily transmitted by *Aëdes aegypti*. A search for *L. icteroides* in 15 cases of yellow fever yielded negative results.

In the meanwhile several experimenters made observations which called in question the opinion that *L. icteroides* was the cause of the true yellow fever of

the Americas, as represented by the disease studied by the commission under Reed. Sellards (7) was unable to demonstrate by serological methods any relationship between a yellow fever outbreak in Parahyba, Brazil, and *L. icteroides* or *L. icterohaemorrhagiae*. Theiler and Sellards (8) and Schüffner and Mochtar (9) compared *L. icteroides* and *L. icterohaemorrhagiae* and obtained strong evidence of their serological identity.

Attempts to transfer *L. icteroides* by means of *Aëdes aegypti* failed in the hands of a number of investigators (Kligler (10), Gay and Sellards (11), Schüffner and Mochtar (9), and Sawyer and Bauer (12)) and they became convinced that this mosquito is not suited to act as intermediate host for *L. icteroides*.

When our experiments were undertaken there seemed to be need for further investigation of the relationships of the "yellow fever" of West Africa, the "yellow fever" of South America not due to a leptospira, the "yellow fever" of the Americas apparently caused by a leptospira, and a hypothetical "yellow fever" in which the etiological factors of the two last mentioned diseases are both present.

Collection of Strains of Yellow Fever Virus

The strains of virus used in our experiments were the French and the Asibi from West Africa and the F. W. from Brazil.

The French strain was given us by Dr. A. W. Sellards and Dr. Max Theiler of the Harvard Medical School. It had been obtained originally from a yellow fever patient in Senegal by Mathis, Sellards, and Laigret (13). During our experiments this strain was indistinguishable from the Asibi strain in its effects on monkeys.

The Asibi strain was sent us from Lagos, Nigeria, by Dr. Henry Beeuwkes, Director of the West African Yellow Fever Commission of the Rockefeller Foundation. It had been obtained in the Gold Coast from an African native during an attack of yellow fever, and had been used by Stokes, Bauer, and Hudson (5) in most of their experiments.

The establishment in our laboratory of a strain of yellow fever virus from South America proved to be difficult. We are indebted to Dr. F. L. Soper and Dr. H. Muench for obtaining for us a large number of specimens of blood from yellow fever patients in Rio de Janeiro. We wish to acknowledge also our special obligation to Dr. Clementino Fraga, Director of the National Department of Health, and to Dr. Sinval Lins, Chief of the Communicable Disease Section of Hospital São Sebastião, for their courtesy in granting Dr. Soper and Dr. Muench

free access to the patients. Specimens of blood and tissues from monkeys experimentally infected with yellow fever were received through the kindness of Dr. H. de B. Aragão of the Oswaldo Cruz Institute in Rio de Janeiro and of Dr. N. C. Davis and Dr. J. H. Bauer of the Yellow Fever Laboratory of The Rockefeller Foundation at Bahia.

The specimens of human blood from Rio de Janeiro were taken from 103 patients having yellow fever or suspected of having it. A citrated specimen from each patient was received and also dried blood from three and clotted blood from four. Among the patients supplying the blood were at least two from whom yellow fever was transmitted to monkeys in Rio de Janeiro. This was accomplished by blood inoculation by Da Cunha and Muniz (14) in the case of F.W., and through the bites of mosquitoes by Aragão (15) in the case of N.M. The blood was drawn on the first day of the disease from 11 patients, on the second from 37, on the third from 53, and on the fourth from one. In one instance the day of disease was not recorded.

As soon as received, the specimens were injected into *M. rhesus* monkeys, in varying amounts, usually intraperitoneally, rarely subcutaneously. Of the 110 specimens, including duplicates, 16 were injected into separate animals. The remaining 94 were divided into 14 groups and pooled, and part of each of the 14 mixtures was injected into one or two monkeys. In some instances animals which showed no symptoms were inoculated later with another specimen as the number of available monkeys was limited. Test injections with a known virus were not given at the end of the observation period to determine whether immunity to yellow fever had been established as we had no yellow fever virus during the early part of the work. The rectal temperature of the monkeys under observation was taken twice each day.

The results from the inoculation of the specimens of human blood sent from Rio de Janeiro were uniformly negative. Most of the monkeys developed no fever. Six had isolated rises of temperature which seemed of no significance. We concluded that it was not practicable to isolate strains of yellow fever from the blood of patients in South America while working at so great a distance from the source of material.

Attempts to bring in a strain of yellow fever already established in monkeys finally met with success. From Rio de Janeiro Dr. Aragão sent us material from monkeys infected with several strains (F.W., N.M., N.M. and J.K., N.M. and D.A., J.K. and R.M.). We received citrated, glycerinated, and dried monkey blood (one specimen of each) under refrigeration; an emulsion of mosquitoes which had fed on an infected monkey; and monkey liver which had been refrigerated (5 specimens), frozen (3), glycerinated (3), dried (2), and one kept at room temperature. These eighteen specimens were prepared and injected into animals

intraperitoneally or subcutaneously. Six of the animals died from causes other than yellow fever. The other animals remained normal except one.

A monkey which had been inoculated with a specimen of frozen liver, F.W. strain, tenth passage in monkeys, showed fever on the seventh day and recovered. After an observation period of 28 days from the date of inoculation this monkey was given a test injection of African yellow fever virus, Asibi strain, and was found to be immune. The strain obtained by bleeding this monkey at the beginning of its fever was carried through many passages in this laboratory, and the F.W. virus used in our experiments was derived from this succession.

Like the African strains, the F.W. strain thus procured could withstand long storage under suitable conditions. At one time the strain was apparently lost, but was reestablished by inoculating a monkey with stored liver tissue. The specimen of liver had been kept continually frozen for 67 days. Between experiments this strain was successfully preserved in storage for varying intervals up to 92 days in monkey blood dried in the frozen state by the methods used by Sawyer, Lloyd, and Kitchen (16) in preserving African strains of yellow fever virus.* We have not yet tested older dried blood specimens of the F.W. strain by inoculation of monkeys.

The material from Bahia, Brazil, consisted of 28 specimens of blood and liver from 20 monkeys experimentally infected with yellow fever of the B.B. strain (27 specimens) and the S.R. strain (1 specimen). These strains had been obtained from yellow fever patients in Bahia, Brazil, by Davis and Burke (17). This material was injected into monkeys and the B.B. strain was established in our laboratory, but the virulence of this strain for monkeys was so low that it seemed unsuitable for our projected cross-immunity experiments.

In the hands of Davis and Burke (17) the B.B. strain has shown a higher degree of virulence. Immunity against African virus was possessed by four of eight animals that developed fever after inoculation by us with the B.B. strain specimens. This adds to the evidence supporting Davis (18) in his conclusion that this strain is immunologically the same as African yellow fever virus.

Bacteriological Examinations

The specimens of citrated human blood received from Rio de Janeiro were tested for bacterial contamination by inoculation of broth or agar slants. Only three specimens produced visible growths and they were caused by three different organisms. A more critical bacterial examination was made by Frobisher (19) of 30 of these specimens of human blood and also of the blood of 16 monkeys infected

* A specimen of African yellow fever virus (French strain) in monkey blood preserved in this way has been tested after one year in storage and found to be highly virulent for *rhesus* monkeys.

with African or South American yellow fever virus and of the liver tissue of 10 monkeys infected with African virus. Cultures were made in a wide variety of media, including the Noguchi lepto-medium, and guinea pigs were inoculated. The results were essentially negative.

Of the specimens of citrated human blood sent us from Rio de Janeiro, 66 were examined also by H. R. Muller and E. B. Tilden (20) at The Rockefeller Institute for Medical Research. Two of these specimens yielded cultures of a spiral organism agreeing with the cultures of *L. icteroides* isolated in yellow fever epidemics by Dr. Noguchi. Both of the specimens containing leptospirae came from severe cases with the symptoms of yellow fever; one of the cases was fatal. Both specimens were among the first 36 examined, which group was included in the 103 specimens inoculated by us into monkeys.

Tests of Monkeys Immunized with American Yellow Fever Virus for Immunity to African Virus

In order to show the relationship of American yellow fever to the African, monkeys were immunized with American yellow fever virus and afterward tested for susceptibility to African virus, and *vice versa*.

In Table I are given the results of the tests of eleven monkeys which had exhibited fever after intraperitoneal inoculation with the F.W. strain of American yellow fever virus. Monkeys which had not shown fever after inoculation with this strain were sometimes found to be immune to African virus and sometimes not. They were all excluded from this experiment as some had probably not been infected.

Three of the monkeys were inoculated a second time with the F.W. strain from 5 to 7 weeks after the first inoculation, in order to make certain that they had become immune to American yellow fever virus. To obtain additional evidence regarding the immunity produced and a better idea of its degree, blood was taken from six of the animals between the 16th and 41st days after the last injection of American virus, and the serum was tested for protective power against African virus. All eleven of the monkeys were tested by intraperitoneal injection of African virus when at least 30 days had elapsed after their last inoculation with American virus, and the high virulence of the African virus used was shown by its effect on control animals. Except when otherwise stated in the table, the period

of observation after the test inoculation was 30 days or longer. Only temperatures of 40°C. or over were accepted as fever unless otherwise stated.

All of the eleven monkeys immunized against American yellow fever virus survived inoculation with a highly virulent strain of African yellow fever virus, and only one showed fever within the period of

TABLE I

Immunity to African Yellow Fever Virus in Monkeys after Infection with American Yellow Fever Virus

Monkey	First inoculation with American virus		Second inoculation with American virus		Protection test of monkey's serum against African virus†	Test of monkey for immunity by inoculation with African virus (Asibi)
	Strain	Febrile period*	Strain	Febrile period*		
B	F.W.	5.5 to 12.0	F.W.	11.0 to 13.0	Protected‡	Immune
C	F.W.	2.5 to 15.0	F.W.	None	Protected	Immune
D	F.W.	2.5 to 9.0	Not inoculated		Protected	Immune
E	F.W.	7.0 & 12.0	Not inoculated		Protected‡	Immune
F	F.W.	7.5 to 9.5§	F.W.	None	Protected	Immune
G	F.W.	3.0§	Not inoculated		Not tested	Immune
H	F.W.	2.0 to 8.5	Not inoculated		Protected‡	Immune
I	F.W.	6.0 to 7.5	Not inoculated		Not tested	Immune‖
J	F.W.	3.0 to 4.5	Not inoculated		Not tested	Immune¶
K	F.W.	4.0§	Not inoculated		Not tested	Immune
L	F.W.	4.0 to 12.0	Not inoculated		Not tested	Immune

* Expressed in days after inoculation.

† Performed with Experiment IV; same methods, same virus (Asibi), regular amount of serum, and same controls (Table IV).

‡ Had one elevation of temperature; Monkey B on 14th day after inoculation, Monkey E on 7th, Monkey H on 8th.

§ Maximum temperature 39.7°C. (Monkey G) or 39.9°C. (Monkey K).

‖ Observed for 24 days only.

¶ Fever 4.0 to 6.0 day. Observed for 12 days only.

observation. The sera of six of these monkeys taken before the test inoculation were tested for power to protect monkeys against the same African strain. All protected against death, although three permitted fever.

Tests of Monkeys Immunized with African Yellow Fever Virus for Immunity to American Virus

As it was a rare exception for a monkey to survive inoculation with fresh virus of the African strains which we used (French and Asibi), it was necessary in our cross immunity tests to use monkeys which

TABLE II

Immunity to American Yellow Fever Virus in Monkeys after Infection with African Yellow Fever Virus

Monkey	First inoculation with African virus		Second inoculation with African virus		Protection test of monkey's serum against American virus (F.W.)	Test of monkey for immunity by inoculation with American virus (F.W.)
	Strain	Febrile period	Strain	Febrile period		
M	Asibi	None	Asibi	None	Not tested	Immune*‖
N	Asibi	None†	Asibi	1.5	Not tested	Immune*
O	French	None†	Asibi	None	Not tested	Immune
P	French	None†	Asibi	None	Not tested	Immune
Q	French	12.5 to 15.0	Asibi	None	Not tested	Immune‖
R	French	6.0 to 7.0	Asibi	4.5	Not tested	Immune
S	Asibi	5.0	French	None	Not tested	Immune
T	Asibi	3.0, 3.5	French	None	Not tested	Immune
U	French	1.5 to 5.0	Not inoculated		Doubtful‡	Immune
Control: C§						Febrile period 2.5 to 15.0

* Had received an injection of the B. B. strain of American virus but, as the control monkey as well as the animal tested showed no reaction, the monkey was reinoculated 14 days later with the F. W. strain.

† Protected by an injection of African immune serum from recovered African natives (Monkeys N, and P) or from immunized Monkey A, Table IV (Monkey O).

‡ Had irregular unexplained rises of temperature from day of inoculation.

§ Monkey C was control for the examination of the sera of Monkeys M, N, O, P, Q, R. The other tests were made with the controls shown in Table V.

‖ Observation period only 20 days (Monkey M) or 21 days (Monkey Q).

had been protected against death by injections of African immune serum or which had received virus attenuated by some method of preservation or by long storage. Nine animals immunized against African virus were available for testing. Particulars with regard to the tests are shown in Table II.

Eight of the monkeys tested had been given a second injection of the African virus from 20 to 48 days after the first as a test of immunity to that virus. The monkeys were tested by inoculation with American virus (F.W. strain) after an interval of between 31 and 39 days from the last injection of African virus, except in one instance in which the interval was 143 days. They were kept under observation for 30 days or more, except when otherwise stated in the table.

Blood was taken from one of the animals before the test inoculation and the serum was tested for protective power against American virus. As the monkey receiving this serum with the virus had irregular fevers not due to the yellow fever virus, it was impossible to know whether an attack of fever was caused by the virus and no conclusion could be drawn, as death does not ordinarily result from the F.W. virus in this laboratory.

All nine of the monkeys immunized against African yellow fever were free from fever following inoculation with the F.W. strain of American virus.

In the cross immunity tests, monkeys immunized against American yellow fever virus resisted African yellow fever virus and, conversely, those immunized with the African virus resisted the American virus.

Protective Power of American Yellow Fever Sera against African Virus and against Leptospirae

A. Sera Taken Soon after Undoubted Yellow Fever

An exceptional opportunity to investigate the relationship of American yellow fever to African yellow fever and to leptospiral jaundice was presented by an epidemic of yellow fever in Rio de Janeiro. Through the courtesy of Dr. H. de B. Aragão and Dr. F. L. Soper we received 15 specimens of serum from 14 persons who had recovered recently from yellow fever in that city. They are listed in Table III under the heading "Recent epidemic," and our findings with regard to these sera are summarized there.

In all the 14 cases a definite diagnosis of yellow fever had been made on clinical grounds. In one case (N.M.) the diagnosis had been proven, for Aragão (15) had transferred the infection to a monkey by means of mosquitoes. In eight cases the attack was described as severe; in six as mild.

Six of the sera were divided in New York and a portion of each was sent to the

TABLE III

Protection by Sera of Persons Recovered from American Yellow Fever against African Yellow Fever Virus and Leptospira icteroides or L. icterohaemorrhagiae

Serum	Time after attack	Protection of monkey against yellow fever virus*		Protection of guinea pig against leptospirae*			Experiment	
		Prevented fever	Prevented death	Pfeiffer phenomenon	Prevented fever	Prevented death	Tests with virus	Tests with leptospira
Recent epidemic:								
N. M.	31 days	+	+	−	−	−	I	A
C. F.	39 days	+	+	−	−	+‡	I	A
D. A.	26 days	−(1.5)†	−(3.5)†	−	−	−	I	A
M. A. (1st specimen)	16 days	+	+	−	−	‖	I	A
F. C.	10 days	+	+	−	−	−	I	A
J. R. S.	85 days	+	+	−	−	−	I	A
G. V.	15 days	−¶	+	−	−	−	II	A
M. R.	49 days	+	+	−	−	−	II	B
M. A. (2nd specimen)	40 days	+	+	−	−	−	II	B
J. M.	20 days	+	+	−	−	+‡	II	B
M. M.	144 days	−(3.5)§	+	−	−	−	III	C
M. J. S.	153 days	−(2.0)	−(4.5)	+	+	+	III	C
R. B. S.	156 days	**	−(4.0)	−	−	−	III	C
J. M.	153 days	−(4.0)§	+	−	††	††	III	C
C. W. G.	47 days	−(2.0)	−(4.5)	+	+	+	IV	C

* The yellow fever virus was of the strains Asibi in Experiments I and IV and French in Experiments II and III. The strains of leptospira used were *Leptospira icteroides*, Brazil 49, in Experiments A and B, and *Leptospira icterohaemorrhagiae*, Rat I, in Experiment C.

† The figure in parenthesis is the number of days from inoculation to the first observation of fever in column "Prevented Fever," and from inoculation to death in column "Prevented Death."

‡ Guinea pig had fever but survived and was found to be immune to a second injection of *L. icteroides*.

§ Temperature rose as high as 40°C. only once.

‖ Guinea pig had fever, but died on second day after inoculation while being bled; too early for characteristic lesions.

¶ Had unexplained occasional single elevations of temperature from the 1st to the 58th day after inoculation.

** No fever observed.

†† Guinea pig died from unknown cause on day following inoculation.

TABLE III—*Concluded*

Serum	Time after attack	Protection of monkey against yellow fever virus*		Protection of guinea pig against leptospirae*			Experiment	
		Prevented fever	Prevented death	Pfeiffer phenomenon	Prevented fever	Prevented death	Tests with virus	Tests with leptospira
Doubtful cases:								
Car. F.	brief	−(1.5)	−(4.0)	−	**	−	III	C
M. D.	brief	−(1.5)	−(4.5)	−	−	−	III	C
H. D.	brief	+	+	−	**	−	III	C
Dj. A.	brief	+	+	‡‡			III	
Former epidemics:								
G. E.	9 years	−(5.0)	+	−	−	−	II	B
C. H. H.	5 years	−(2.0)	−(5.0)	−	−	−	II	B
D. O. L. (1st test)	23 years	−(2.0)	−(9.0)	−	−	−	II	B
D. O. L., double amount		−(4.0)	+				IV	
I. J. K., 0.4 of amount	8½ years	+	+	‡‡			III	
P. S. R., double amount	30 years	−(2.0)	−(4.5)	‡‡			IV	

‡‡ No serum remained for test.

West African Yellow Fever Commission of the Rockefeller Foundation in Lagos. The results of the tests made by the Commission in Africa were reported by Hudson, Philip, and Davis (21). They found that the sera of N.M., C.F., M.R., M.A., and J.M. protected monkeys against injection of African yellow fever virus (Cases 16, 15, 12, 14, and 17 in their series). One of the sera, however, that of D.A. (Case 13 in their series) failed to protect either of two monkeys against the injection of the virus. Four of these sera, those of N.M., C.F., M.A., and J.M., were tested in guinea pigs against *L. icterohaemorrhagiae*, and in every case the Pfeiffer phenomenon was absent and the animal died of leptospirosis. The results of the protection tests in monkeys were in agreement with those of the tests we performed independently in New York with the same sera, and their Pfeiffer tests gave results consistent with those we obtained using different strains of leptospira.

Explanation of Tables III and IV. The results of tests with the sera of persons who had recovered from American yellow fever for protective power against African yellow fever virus and against *L. icteroides* or *L. icterohaemorrhagiae* are shown in Tables III and IV. The results of the tests of the sera themselves are in Table III and those of the corresponding control tests are in Table IV. The tests for protection against yellow fever virus were made in four separate experiments

TABLE IV

Control Tests in the Experiments Recorded in Table III

Serum	Time after attack	Protection of monkey against yellow fever virus		Protection of guinea pig against leptospira			Experiment	
		Prevented fever	Prevented death	Pfeiffer phenomenon	Prevented fever	Prevented death	Tests with virus	Tests with leptospira
Recovered African native, K. S.	10 mos.	+	+	−	−	−	I	B
Recovered African native, K. Owe.	9 mos.	−(6.5)	+	−	−	+*	II	B
Recovered African native, K. Ot.	22 mos.			−	†	−		C
Monkey A, immunized to African virus		+	+	−	−	−	II	B
Normal *rhesus* monkey, double amount		−(2.5)**	−(4.5)	−	−	−	IV	B
Anti-icteroides, double amount		−(2.5)	−(6.5)	+	‡	‡	I	A
Anti-icteroides, 1:10 dilution				+	+	+		C
Anti-icteroides, 1:100 dilution				+	§	§		B
Normal horse				−	−	+*		A
Normal human, double amount		−(1.5)	−(4.5)	−	−	−	I	A
Normal human, double amount		−(1.5)	−(4.0)	−	−	−	II	C
Normal human, double amount		−(1.5)	−(3.5)				III	
Normal human, double amount		‖	−(6.0)				IV	

* Guinea pig had definite febrile attack but survived and was found to be immune to a second injection of *L. icteroides*.

† No fever observed. Death from leptospiral jaundice 5 days after inoculation.

** Temperature rose only to 39.9°C.

‡ Guinea pig had no fever for 17 days, then developed fever and died 6 days later of pneumonia and peritonitis, but without lesions suggesting leptospiral jaundice.

§ Guinea pig had fever and died of peritonitis on the seventh day. No lesions suggestive of leptospiral jaundice.

‖ No fever was observed.

TABLE IV—*Concluded*

Serum	Time after attack	Protection of monkey against yellow fever virus		Protection of guinea pig against leptospira			Experiment	
		Prevented fever	Prevented death	Pfeiffer phenomenon	Prevented fever	Prevented death	Tests with virus	Tests with leptospira
No serum, full amount of virus		−(1.5)	−(5.0)				I	
No serum, 0.1 amount of virus		−(2.0)	−(4.5)				I	
No serum, 0.01 amount of virus		−(10.0)	−(11.0)				I	
No serum, full amount of virus		−(6.0)	+¶				II	
No serum, 0.1 amount of virus		−(2.0)	−(4.0)				II	
No serum, 0.01 amount of virus		‖	−(8.5)				II	
No serum, 0.1 amount of virus		−(3.0)**	−(5.0)				III	
No serum, full amount of virus		−(2.5)††	−(4.5)				IV	
No serum, 0.01 amount of virus		−(2.5)	−(4.0)				IV	
Salt solution				−	−	−		A
Salt solution				−	−	−		B
Salt solution				−		‡‡		C

¶ Monkey survived, although it had received no serum.

†† Temperature rose only to 39.8°C.

‡‡ Guinea pig died on night following inoculation.

designated by the Roman numerals I, II, III, and IV, and the tests for protection against leptospirae were made in three experiments, A, B, and C. In order to show which controls belong to each experiment the numbers and letters identifying them are given in the column headed "Experiment."

The sera tested were injected intraperitoneally into *M. rhesus* monkeys in amounts of 1.5 cc. per kilogram of body weight except where otherwise stated in the column headed "Serum." The expression "double amount" means that 3.0 cc. per kilogram was given, and "0.4 amount" means 0.6 cc. per kilogram. These variations in amount of serum do not apply to the tests with leptospirae. The "time after attack" was measured from the onset of the disease to the time of bleeding to procure the serum.

Inoculations of monkeys with African yellow fever virus were given subcutaneously six hours after the serum had been given intraperitoneally. The amount of the virus injected was 0.3 cc. per kilogram of body weight in experiments I, II, and III, and 0.2 cc. per kilogram in Experiment IV.

The temperatures of the monkeys were taken twice each day. To eliminate the personal equation as far as possible, only temperatures of 40°C. or above were considered as signifying fever except when otherwise stated in the tables. In the column headed "Prevented fever" the plus sign (+) signifies that the monkey had no fever and remained well during an observation period of 30 days after inoculation. The minus sign (−) in this column indicates that fever developed, and was probably due to yellow fever, unless explained in a footnote. In the column "Prevented death" a plus sign (+) shows that the animal did not die. Survival was not necessarily due to the action of the serum, for very rarely recoveries follow infection with the strains of African virus in use, as for example in the case of one of the controls receiving no serum. A minus sign (−) in this column signifies that the monkey died of yellow fever. The diagnosis in all fatal cases of yellow fever was determined by finding characteristic lesions on postmortem examination and on histological examination of the tissues.

In the tests of sera for their power to protect guinea pigs against leptospirae a strain of *L. icteroides*, Brazil 49, was used in Experiments A and B, and one of *L. icterohaemorrhagiae*, Rat I, in Experiment C. Both these strains were given us by H. R. Muller, of The Rockefeller Institute for Medical Research. Strain Rat I had been isolated recently from a wild rat of New York.

In the Pfeiffer tests 1 cc. of the serum to be tested was mixed with 1 cc. of an active culture of leptospirae, and 1 cc. of the mixture was injected immediately into the peritoneal cavity of a guinea pig. Fluid was withdrawn from the peritoneal cavity of the guinea pig 30, 60, 90, and 120 minutes after inoculation and examined under the dark-field microscope. If the Pfeiffer phenomenon was absent, the result was recorded with a minus sign (−) under "Pfeiffer phenomenon." In the cases in which the record shows a plus sign the Pfeiffer phenomenon was present and unmistakable.

The guinea pigs which had been inoculated in the Pfeiffer tests were kept under observation to determine whether they were protected against experimental leptospiral jaundice by the sera injected. In the column "Prevented fever" a plus sign means that the guinea pig remained free from fever and a minus sign that it developed fever. In the column "Prevented death" a plus sign indicates survival, not always due to the serum, and a minus sign means death from experimental leptospiral jaundice. Footnotes explain the irregular results. The diagnosis of leptospiral jaundice in the guinea pig was made by the observation of the jaundice, extreme hemorrhages, and other characteristic lesions, and usually by the observation of leptospirae in the tissues or body fluids.

The tests of the sera of the fourteen persons who had recovered recently from yellow fever in the epidemic in Rio de Janeiro gave the following results:

10 persons—the serum protected against African yellow fever virus, but not against leptospirae (tests with *L. icteroides* in 8 instances and *L. icterohaemorrhagiae* in 2).

2 persons—the serum protected against leptospirae but not against African yellow fever virus (both tests with *L. icterohaemorrhagiae*).

2 persons—the serum protected against neither African yellow fever virus nor leptospirae (tests with *L. icteroides* in one instance and *L. icterohaemorrhagiae* in the other).

The results with the second and third groups of the sera in the above classification were clear cut and regular. Those with the first group showed a few irregularities in the tests in guinea pigs, as is explained in the notes accompanying Table III.

In two cases, in which the guinea pigs died too soon for diagnosis, the definitely negative Pfeiffer test may be accepted as indirect evidence of the lack of protective power in the serum, since in the other cases negative Pfeiffer tests were followed by failure to protect and positive Pfeiffer tests by protection of the guinea pigs. Two guinea pigs inoculated with serum and leptospirae developed fever and recovered. That occasional recoveries are to be expected after inoculation with the strain of *L. icteroides* used, in the absence of a protective serum, is shown by the behavior of a control guinea pig which received the same strain with normal horse serum and recovered (Table IV).

The two species of leptospirae, *L. icteroides* and *L. icterohaemorrhagiae*, appear to be so closely related that they may be used interchangeably in immunological tests for leptospiral jaundice, as in this investigation of ours, and we have already referred to the work of investigators who obtained strong evidence of their serological identity. It will be noticed in Table IV that anti-icteroides serum, even when diluted, gave positive Pfeiffer reactions with both species of leptospira. This serum had been prepared by The Rockefeller Institute by immunizing a horse against a number of strains of *L. icteroides*.

The absence of protective power against African yellow fever virus in four of the sera from recent cases of yellow fever in Rio de Janeiro (D.A., M.J.S., R.B.S., and C.W.G.) is in our opinion strong evidence against the presence of yellow fever in these cases, in view of the evidence already presented showing that African yellow fever and American yellow fever are immunologically the same. That some of the

sera sent from Rio de Janeiro should be from cases other than of yellow fever casts no reflection on the ability of the diagnosticians, for in many cases it is impossible to distinguish by symptoms between yellow fever and leptospiral jaundice, and mild yellow fever may simulate a number of infections.

As evidence of the lack of protective power in these sera we have the results following the inoculation of one monkey in each case, and the confirmatory observations, already mentioned, of Hudson, Philip, and Davis (21) in the case of D.A. The evidence from the inoculation of one monkey in each case seemed conclusive, however, as there was no suggestion of even partial protection by amounts of serum varying from 3.2 cc. to 5.9 cc. according to the weights of the animals.

Observations which bear on the reliability of such negative protection tests have been tabulated by Hudson (22) and relate to sera of 23 West African natives who had recovered recently from yellow fever during observed epidemics. Of 28 monkeys, each of which had received 4 cc. of serum and 0.25 cc. to 1.0 cc. of blood virus (Asibi strain), only one succumbed to yellow fever. The possibility of error of diagnosis in the case of the person supplying the one negative serum cannot be completely ruled out. Hudson gives the estimated number of spontaneous recoveries of *rhesus* monkeys after inoculation with the Asibi strain of virus as about two per cent.

Two of the sera from Rio de Janeiro (M.J.S. and C.W.G.) had strong protective power against *L. icterohaemorrhagiae*, and in one of these cases (C.W.G.) the occurrence of a definite relapse after discharge from the hospital is against the diagnosis of yellow fever and suggestive of leptospiral jaundice, which so commonly has an after-fever. H. R. Carter (23), after a wide experience with yellow fever, said of that disease, "I have never seen a relapse, but other men have. They must be rare." Although the possibility cannot be entirely excluded that the protective substances in either serum were due to an earlier attack of leptospiral jaundice, it is highly probable that in both cases the illnesses taken for yellow fever were in reality leptospiral jaundice (Weil's disease).

M.J.S. had a mild attack and was discharged from the hospital as cured on the seventh day after the onset, according to data published by Barreto (24).

C.W.G. came to New York after his recovery and permitted us to draw an additional specimen of blood. He also supplemented the information which had been sent us by Dr. F. L. Soper. The illness of C.W.G. was characterized by high fever, headache, great weakness, vomiting, backache beginning with the

second day, moderate albuminuria from the fourth day, and jaundice from the seventh. During the first four days the symptoms were such that the physicians were of the opinion that the disease was not yellow fever, but on the seventh day a definite diagnosis of yellow fever was made. The patient was allowed to get up on the 13th day and to leave the hospital on the 15th. On reaching home he again developed high fever and headache. On the 17th day he was able to leave his bed. Convalescence was slow, and he was still distinctly jaundiced on the 48th day, when he visited our laboratory in New York. In Rio de Janeiro he worked at the water front and docks in places where there were many rats. An investigation of this case in Brazil has been published by R. A. Warner (25).

As the sera from most of the 14 persons in Brazil who had recently had symptoms like those of yellow fever possessed strong protective power against African yellow fever virus, the conclusion seems justified that the yellow fever of America is the same disease as that of Africa. The same conclusion has been reached by Theiler and Sellards (26), Hudson, Bauer, and Philip (27) and Hudson, Philip, and Davis (21) as the result of their protection tests with American sera.

B. Sera Taken Soon after Suspected Yellow Fever

Four of the sera sent us came from a town in southern Brazil, and were from cases in which yellow fever was only suspected. In each case the illness was mild and a diagnosis could not be made from the symptoms. These specimens were tested with the results recorded under the heading "Doubtful cases" in Table III Two of the sera protected completely against yellow fever and two did not protect. at all. One of the sera which protected against the virus and the two others were tested for power to protect against *L. icteroides*, and none protected. We were evidently dealing with two cases of yellow fever, and two cases of infection other than yellow fever or leptospiral jaundice.

C. Sera Taken Long after Yellow Fever

The testing of the sera from recent cases of yellow fever at Rio de Janeiro indicated that the Brazilian disease was the same as that of Africa. The assumption was also that it was identical with the disease studied by Reed in Cuba and suppressed by Gorgas in Panama. The fact, however, that the ocean passage between Senegal and Brazil may be as short as six days permits of the possibility of the transport of the African yellow fever to Brazil, although there is no evidence of this at the present time. Fortunately the relationship of the present yellow fever of Africa and Brazil to the historic yellow fever of America

need not be left to speculation. Direct evidence has been obtained by the examination of the sera of persons who passed through attacks of American yellow fever many years ago.

Serological evidence of the unity of the American yellow fever of the past and the African yellow fever of the present has been secured by Bauer and Hudson (28). Two sera obtained 23 years after attacks of yellow fever in the Panama Canal Zone and one taken 26 years after an attack in Tampico, Mexico, protected monkeys against African yellow fever. A fourth serum taken 26 years after yellow fever in New Orleans failed to protect. The tests were made in duplicate.

The results of the tests of five sera from persons who had had yellow fever in the Americas many years ago are given under the heading "Former epidemics" in Table III. The particulars regarding the sources of these sera are as follows:

G.E. had yellow fever in La Union, Salvador, in July, 1919, soon after arriving from Honduras. The attack was very severe and lasted 14 days. It was characterized by much vomiting, heavy albuminuria, and jaundice. According to the patient there were other cases of yellow fever in La Union and in Honduras at the time. Blood was obtained for us by Dr. J. E. Elmendorf, Jr., and information regarding the case was supplied by Dr. Peralta Lagos. The interval between the attack and the bleeding was 9 years and 3 months.

C.H.H. had yellow fever in Aracajú in the State of Sergipe, Brazil, in May, 1923, according to his statement. We are indebted to Dr. B. E. Washburn and Dr. Hargreaves of Kingston, Jamaica, for obtaining blood. The blood was drawn 5 years after the attack.

D.O.L. had a very severe attack of yellow fever in the city of Panama in June 1905. He was attended by several doctors who had had a large experience with yellow fever, including Colonel W. C. Gorgas, and Dr. H. R. Carter. We are indebted to Dr. W. E. Deeks and Dr. R. C. Connor for putting us in touch with D.O.L., and to Captain J. W. Smith of the Medical Corps of the U. S. Army for drawing a specimen of his blood. The interval between the attack of yellow fever and the drawing of blood was 23 years and 5 months.

I.J.K. contracted yellow fever in Morropón, Peru, in April, 1920, during an epidemic, and had a mild attack. He spent a few months in West Africa in 1926. In his case the interval between the disease and the bleeding was 8 years and 7 months. A different specimen of serum of I.J.K. was tested by Hudson, Bauer, and Philip (27) and found to protect against African virus, Asibi strain.

P.S.R. had yellow fever in Havana, Cuba, in May, 1899, 30 years before the specimen of serum was taken.

The results obtained in these tests were as follows:

Serum taken 8½ years after an attack of yellow fever in Peru protected a monkey completely against African yellow fever. Serum taken 9 years after yellow fever in Salvador permitted the appearance of fever in the monkey but prevented death. A specimen obtained 23 years after an undoubted attack of yellow fever in Panama failed to protect when injected in the usual amount, but prevented death, while permitting fever, when used in double quantity. The same amount of normal human serum failed to prevent the death of a control animal in this experiment and in each of three other experiments (Table IV). A serum taken five years after yellow fever in Northern Brazil failed to protect. The evidence with regard to the diagnosis in this case was meager. Serum taken 30 years after yellow fever in Cuba did not protect.

The results obtained in these tests and those of the other investigators cited show the historic yellow fever of America to have been the same as the present African yellow fever.

Protective Power of African Yellow Fever Sera against American Virus

If we were right in our conclusion, derived from observation of the protective power of American sera against African virus, that the yellow fever of America is the same as that of Africa, then the sera of persons who have recovered from yellow fever in Africa should protect likewise against American yellow fever virus.

To demonstrate this by experiment proved difficult owing to the low virulence of the F.W. strain of American yellow fever virus for monkeys, as the strain exists in this laboratory. Only twice in our experience has death resulted from the inoculation of a monkey with the F.W. strain. In using this strain in protection tests it was necessary, therefore, to base our conclusions on the presence or absence of fever in the experimental animals after inoculation. Under these circumstances the results of the tests would of necessity be inconclusive in relation to the individual specimen of serum, but they should permit conclusions with regard to a group of sera when compared with an adequate series of control tests.

In Table V are shown the results of tests of the sera of six natives of the Gold Coast of West Africa. All of these persons had had yellow fever in observed yellow fever epidemics. We are indebted to Dr. Henry Beeuwkes, Director of the West African Yellow Fever Commission of the Rockefeller Foundation, for these sera.

TABLE V

Protection by Sera of Persons Recovered from African Yellow Fever against American Yellow Fever Virus

Serum	Time from attack to bleeding	Protected monkey from fever after inoculation with American yellow fever virus, F.W. strain*	Result of later test inoculation of monkey with African virus, Asibi strain	Results of other tests of the sera
African natives:				
K. Ot.	22 mos.	+	No reaction	Did not protect against *L. icteroides* (Table IV)
K. B.	23 "	+	No reaction	Protected against African yellow fever virus (Asibi)†
K. Owir.	20 "	+	Survived‡	
J. O.	19 "	+	No reaction	
K. N.	30 "	+	No reaction	
T. C.	33 "	+§		
Controls:				
American, recent recovery, M. A.	40 days	+	No reaction	Protected against African yellow fever virus (Table III)
American, recent recovery, C. W. G., double amount	47 "	−(11)‖	Survived‡	Protected against *L. icterohemorrhagiae*, but not African yellow fever virus (Table III)
Anti-icteroides, double amount		−(3.5)‖	No reaction	Protected against *L. icteroides* and *L. icterohemorrhagiae*, but not African yellow fever virus (Table IV)
Normal human, double amount		−(4.0)‖	No reaction	Similar specimens in Table IV

* The F. W. strain showed low virulence for monkeys and did not cause any deaths during this experiment.

† Tested in a later experiment with controls. Monkey showed fever but was protected against death.

‡ Had brief fever reaching 40°C. but recovered.

§ Had no fever but died on 25th day of observation from cause other than yellow fever.

‖ Fever indefinite or absent. The highest temperature of the monkey in the case of C. W. G. was 39.8°C.; in the case of the anti-icteroides serum, 39.7°C.; in the case of the normal human serum, 39.9°C.; in the case of the small dose of virus, 39.8°C.

TABLE V—*Concluded*

Serum	Time from attack to bleeding	Protected monkey from fever after inoculation with American yellow fever virus, F.W. strain*	Result of later test inoculation of monkey with African virus Asibi strain	Results of other tests of the sera
Controls—*Continued:*				
Normal *rhesus* monkey, double amount		−(4.0)	No reaction	Similar specimens in Table IV
No serum, full amount of virus		−(4.0)¶		
No serum, 0.01 amount of virus		−(3.5)‖	No reaction	

¶ Died from dysentery on 28th day after inoculation.

The methods of performing these tests and tabulating the results are in general the same as in the previous experiments (Tables III and IV). The regular amount of serum injected into the monkeys was 1.5 cc. per kilogram of body weight, and the double amount was 3.0 cc. The amount of virus-bearing blood injected was uniformly 0.4 cc. per kilogram, and it was injected subcutaneously six hours after the serum had been given intraperitoneally.

The results of the tests, though clearly unreliable in the individual case on account of the low virulence of the F.W. strain of virus used, show that the African sera as a group possessed protective power against American yellow fever virus. In no case did fever appear in a monkey which had received one of these six sera. The three control animals which received the full amount of virus without serum or after normal human or monkey serum all showed rises of temperature to 39.9°C. or over at the time when fever would be expected as the result of the inoculation. These observations are in agreement with the stronger evidence obtained by testing American sera against African virus.

Differences between the American and African Strains of Yellow Fever in Their Virulence for Monkeys

The two African strains we used in our experiments (French and Asibi) were similar to each other in virulence for monkeys, but they

differed markedly in this respect from the one American strain (F.W.). To facilitate comparison, we have summarized our experience with the inoculation of *rhesus* monkeys with the Asibi and F.W. strains.

In doing so we have considered only those cases in which the inoculum was blood taken on the first day of fever from monkeys experimentally infected with yellow fever in this laboratory. The amount of blood was not less than 0.1 cc., expressed as undiluted whole blood. If citrated blood was injected, the specimen was not over 24 hours old, and, if dried blood was used, it was prepared by drying in vacuum in the frozen state and was not over 100 days old. In fatal cases the animals were allowed to die or were killed when moribund.

Of 24 monkeys inoculated with African virus of the Asibi strain, all came down with yellow fever and only one survived. Of 20 monkeys receiving American virus of the F.W. strain, 15 developed fever and two of these died. These two, Monkeys V and W, had received dried blood 94 and 63 days old, respectively.

In the cases of those animals that showed temperatures of at least 40°C., the incubation periods for the Asibi strain were, minimum 1.5 days, maximum 6, and average 2.7; for the F.W. strain, minimum 1.5 days, maximum 12, and average 4.3. The intervals between inoculation and death were, for the animals inoculated with the Asibi strain, minimum 2.5 days, maximum 10.5, and average 5. The two animals which died after receiving the F.W. strain did so 4.5 and 8 days after inoculation.

The Asibi strain of African virus proved to have a much higher virulence for monkeys than the F.W. strain of American virus. The former caused death in 23 of 24 monkeys inoculated, and the latter in only 2 of 20. It does not follow, however that there is a similar difference between these strains in their virulence for man, nor that these strains are representative in virulence of the African and American strains in general. Dr. N. Paul Hudson tells us that the figures of the laboratory of the West African Yellow Fever Commission of the Rockefeller Foundation showed, up to the end of 1928, that there was a marked variation in the virulence of African strains for *Macacus rhesus*. A strain (H.P.) obtained from a fatal case in a European killed only one-third of the monkeys inoculated with blood drawn from infected monkeys at the time of fever. The mortality was low also when this strain was transmitted from animal to animal by mosquitoes.

Comparison of the Lesions Produced by African and American Strains of Yellow Fever Virus

The gross and microscopic lesions produced by the Asibi and French strains of African yellow fever virus in our monkeys were in general those described by Hudson (29) for experimental yellow fever as produced in *M. rhesus* by the Asibi strain. The two animals (Monkeys V and W) which died as the result of inoculation with the F.W. strain of American virus showed gross and microscopic lesions such as are produced by the African strains. One animal (Monkey X) killed during a mild attack following inoculation with the F.W. strain showed only very slight changes.

Da Cunha and Muniz (30) speak of the varying extent of the liver lesions in monkeys after inoculation with a Brazilian strain of yellow fever virus. In some animals they found extensive necrosis of the hepatic cells and none in others. Although they found that their virus possessed less virulence than an African strain, it was sufficient at times to cause early death with extensive lesions like those following inoculation with the African strain.

CONCLUSIONS

1. The yellow fever now in South America, the present yellow fever of Africa and the historic yellow fever of Panama and other American countries are the same disease. This conclusion is based on cross immunity tests in monkeys with strains of yellow fever virus from Africa and Brazil and on tests of sera from 25 persons, who had recovered from yellow fever in various places and at various times, for the power to protect monkeys against African or Brazilian virus strains.

2. Cases of leptospiral jaundice (Weil's disease) were present among those diagnosed as yellow fever in the recent epidemic in Rio de Janeiro. This is shown by the isolation of cultures of leptospirae from the blood of two patients by H. R. Muller and E. B. Tilden of The Rockefeller Institute, and by the demonstration by us of protective power against leptospirae and absence of protective power against yellow fever virus in the sera from two persons after recovery. The isolation of leptospirae by Noguchi and other investigators from the

blood of occasional patients in past epidemics of yellow fever in a number of American countries indicates that leptospiral jaundice was present then as well and was diagnosed clinically as yellow fever.

3. The absence of protective power against leptospirae shown by the Brazilian sera which protected against yellow fever virus and the absence of protective power against yellow fever virus in the sera that protected against leptospirae point to the probability that American yellow fever is not the combined effect of leptospirae and yellow fever virus. The position of *L. icteroides*, isolated by Noguchi during yellow fever epidemics, now appears to be not that of a secondary invading microorganism in cases of virus yellow fever, but that of the incitant of a form of infectious jaundice, sometimes fatal, often coincident in its appearance with typical yellow fever and apparently indistinguishable from it clinically. This leptospiral disease has not hitherto been separated from true yellow fever. Noguchi's discoveries become, therefore, of the greatest significance in respect to the epidemiology and causation of yellow fever and of infectious jaundice, previously confused one with the other. In all outbreaks of supposed yellow fever hereafter the existence of the two kinds of jaundice, one due to yellow fever virus and the other to leptospirae will have to be taken into account. Only the former probably is spread by mosquitoes and requires anti-mosquito measures for its control.

4. The only difference observed by us between the American and African strains of yellow fever virus was a pronounced difference in virulence for monkeys. The virulence of the two African strains studied was very high while that of the one American strain was highly variable and usually low.

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